

STRUCTURAL REQUIREMENTS FOR STEROL REGULATORY ELEMENT-BINDING PROTEIN CLEAVAGE IN FISSION YEAST

**By
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Abstract

Cells can respond rapidly to changes in their cellular environments such as oxygen levels, and transcription factors play a critical role in response to such changes to achieve homeostasis. Sterol regulatory element-binding proteins (SREBPs) are membrane-bound transcription factors that serve as master regulators of cholesterol and fatty acid homeostasis. Mammalian SREBPs are proteolytically activated and liberated from the membrane by Golgi Site-1 and Site-2 proteases. However, fission yeast SREBPs, Sre1 and Sre2, employ a different mechanism that genetically requires the Golgi defective for SREBP cleavage (Dsc) E3 ligase complex for cleavage activation.

In this thesis, I defined structural requirements for fission yeast SREBP cleavage. I isolated a collection of cleavage mutants in Sre2 C-terminus and identified a novel SREBP cleavage motif, the glycine-leucine motif. I revealed an extensive conservation of this motif in at least 20 SREBP homologs of ascomycete fungi using defined bioinformatic criteria, including the human opportunistic pathogen *Aspergillus fumigatus* and *Candida albicans* where SREBPs are required for fungal pathogenesis. I also demonstrated functional conservation of this motif in Sre1 as required for adaptation to low oxygen. Further, I identified potential mechanisms for degradation of Sre2 C-terminus by the Dsc E3 ligase and the proteasome. Importantly, I have established Sre2 as the first physiological substrate for dissecting Golgi E3 ligase-dependent protein processing. Therefore, Sre2 can serve as a model substrate and gateway for understanding Golgi protein degradation and processing. Further study into the mechanism of Dsc E3 ligase-dependent SREBP processing entails broad implications for cellular adaptation to

low oxygen and sterol conditions and may enable the development of novel antifungal therapeutics.

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Chapter 1

Introduction

Introduction

Cells must be able to respond rapidly to changes in their cellular environments such as chemicals, gases and macromolecules. Transcription factors play a critical role in response to such changes to maintain homeostasis. Sterol Regulatory Element-Binding Proteins (SREBPs) are membrane-bound transcription factors that serve as master regulators of cholesterol and fatty acid synthesis in mammals (Brown and Goldstein, 1998). Recent research from our group and others has expanded the roles of SREBPs beyond sterol regulation and beyond the mammalian system (Bien and Espenshade, 2010; Hughes et al., 2005; Shao and Espenshade, 2012). In this chapter, I sought to provide a brief history of SREBPs, as well as highlight recent advances in our understanding of SREBP cleavage in fungi, and its implications for hypoxic adaptation, protein quality control in the Golgi apparatus, and fungal pathogenesis.

Mammalian SREBP pathway

Cellular lipid homeostasis is regulated by a class of transcription factors designated sterol regulatory element-binding proteins (SREBPs) (Figure 1.1) (Brown and Goldstein, 1998). In the human genome, two genes code for the three SREBP isoforms: SREBP-1a, SREBP-1c and SREBP-2, which belong to a subfamily of basic helix-loop-helix (bHLH) leucine zipper proteins. SREBP-1a regulates all SREBP target genes, while SREBP-1c and SREBP-2 preferentially regulate genes required for fatty acid and cholesterol synthesis respectively (Espenshade and Hughes, 2007). To activate target gene expression, the SREBP-Scap complex is transported via COPII vesicles from the endoplasmic reticulum (ER) to Golgi (Figure 1.1). In mammalian cells, following sterol-regulated transport to the Golgi, SREBPs are activated through sequential cleavage by the Golgi-resident Site-1 protease and Site-2 protease (Figure 1.1) (Brown and Goldstein, 1997, 1999a).

Specifically, Site-1 protease cleaves in the SREBP luminal loop, splitting the molecule in half. This first cleavage event enables the Site-2 protease zinc metalloprotease to cleave within the first transmembrane segment (TM1) and release the soluble SREBP N-terminal transcription factor from the membrane (Rawson et al., 1997). Mammalian SREBP is cleaved sequentially by the Site-1 protease in the ER luminal loop, before it can be cleaved by the Site-2 protease within TM1 to release functional transcription factor domain (Brown and Goldstein, 1999b).

Site-1 protease belongs to the subtilisin/kexin-like protease family, required for cleaving many proproteins to their active form (Seidah, 2011; Seidah et al., 1998). Subtilisin/kexin-like proteases typically cleave after lysine or arginine and dibasic

sequences (Seidah, 2011; Seidah et al., 1998). Cleavage of mammalian SREBP-2 by Site-1 protease occurs at a luminal RXXL sequence, which in the case of human SREBP-2 cleavage occurs at a leucine-serine bond in the ER lumen (Duncan et al., 1997). More generally, proteases in the subtilisin/kexin-like family cleave after the consensus R-X-(R/K)-R sequence, where X is any amino acid except cysteine (Steiner, 1998).

Following Site-1 protease cleavage, Site-2 protease (S2P) cleaves SREBP-2 at a membrane-embedded leucine-cysteine bond (Duncan et al., 1998). Further domain-swapping studies reveal a cleavage requirement for an asparagine-proline sequence in the middle third of the transmembrane segment (Ye et al., 2000b). The mechanism by which the asparagine-proline sequence facilitates SREBP-2 cleavage is not known, but the favored model suggests a possible conformational change of the SREBP-2 to unwind the transmembrane alpha-helix for access to Site-2 protease (Ye et al., 2000a). In mammals, S2P is essential for membrane release and SREBP activity. This S2P-mediated intramembrane cleavage is an example of regulated intramembrane proteolysis (RIP), with the transcription factor ATF6 demonstrated to be another substrate of S2P responsible for ER stress resolution (Ye et al., 2000c).

Fungal SREBP pathway

The criteria for definition of SREBPs as transcription factors lie in the two predicted transmembrane segments and the unique tyrosine residue in the bHLH DNA binding domain (Figure 1.2). Based on these criteria, SREBPs are also conserved in diverse fungi in addition to animals, but not in bacteria, plant or archaea kingdoms (Figure 1.3). SREBP is conserved in both Basidiomycota and Ascomycota, the two major fungal phyla. It is interesting that under Ascomycota phylum, SREBP is conserved in the fission yeast *Schizosaccharomyces pombe* but not the more popular model organism budding yeast *Saccharomyces cerevisiae* (Figure 1.3). Thus, our group chose to establish the fission yeast as a model to understand SREBP proteolytic activation and regulation in fungi.

Insights from this simple single-celled eukaryote can be gleaned and translated to a more physiologically relevant organism to human health, such as cultured mammalian cells. Studies from our group thus far have revealed novel modes of regulation for SREBP in fission yeast: at the level of regulated SREBP cleavage, protein stability post-SREBP cleavage, and modeling the SREBP feedback loops upon signal sensing (Lee et al., 2007; Stewart et al., 2012; Stewart et al., 2011; Porter et al., 2012; Porter et al., 2010). This thesis focuses on the mechanism of regulated SREBP cleavage in fission yeast.

Fission yeast SREBPs

Fission yeast *Schizosaccharomyces pombe* contains two SREBP homologs: Sre1 and Sre2 (Figure 1.2). Sre1 is an oxygen-regulated transcription factor that is cleaved under low oxygen to promote adaptation to hypoxia (Hughes et al., 2005). Sre1, like the mammalian counterpart, contains a C-terminal domain that binds to Scp1, the homolog of mammalian Scap that also senses sterol levels (Hughes et al., 2008; Hughes et al., 2005). In this system, Sre1 senses oxygen by regulated Scp1 binding as a surrogate for cellular sterol level, because sterol production is highly oxygen consumptive (Hughes et al., 2005; Todd et al., 2006). In contrast, Sre2 lacks the C-terminal regulatory domain and bypasses sterol or oxygen regulation for cleavage; therefore, Sre2 cleavage occurs in the presence of oxygen, and is constitutive and unregulated (Hughes et al., 2005). Fission yeast, like all other ascomycete fungi, lacks an identifiable Site-2 protease homolog (Figure 1.4), suggesting a different mechanism for SREBP cleavage (Espenshade and Hughes, 2007).

Genetic evidence revealed that both Sre1 and Sre2 cleavage requires a multi-subunit Golgi-resident E3 ligase, which we named Dsc (defective for SREBP cleavage) (Stewart et al., 2012; Stewart et al., 2011). The Dsc E3 ligase, composed of Dsc1 through Dsc5, is a stable membrane complex containing the RING-domain E3 ubiquitin ligase Dsc1 (Stewart et al., 2012). The Dsc E3 ligase contains structural domains involved in the ubiquitin/proteasome system (UPS), and is analogous to its homologs in budding yeast and mammalian cells (Lloyd et al., 2013). This parallel is strengthened by additional genetic studies for genes required for yeast SREBP cleavage, which revealed *dsc5* and *dsc6*. Dsc6 codes for Cdc48, an AAA-ATPase that binds to the Dsc E3 ligase

through the ubiquitin regulatory X (UBX) domain of Dsc5 (Stewart et al., 2012).

Previous studies indicate that the mechanism of cleavage is the same for Sre1 and Sre2 (Stewart et al., 2012; Stewart et al., 2011), except that ER exit of Sre1 is regulated by sterols and oxygen and Sre2 exits the ER constitutively (Hughes et al., 2005; Porter et al., 2012).

Our working model for Sre1 cleavage is as follows (Figure 1.5): (1) Sre1 moves from the ER to Golgi under low oxygen or sterol conditions; (2) Sre1 binds to the Dsc E3 ligase; (3) SREBP is ubiquitinated by the E2 ubiquitin-conjugating enzyme Ubc4 and E3 ligase Dsc1; (4) SREBP is subsequently cleaved by an unidentified protease releasing the N-terminal transcription factor domain from the membrane.

Dsc E3 ligase: role in Golgi protein degradation

The ability of the cell to maintain constant flux of cargo proteins and segregation of misfolded proteins in the secretory pathway is termed “protein quality control” (Ellgaard and Helenius, 2003). These processes serve to remove defective proteins that might otherwise be detrimental to the health and viability of eukaryotic cells. Dsc E3 ligase represents a potential protein machine for Golgi protein quality control or degradation, because it is homologous to the Hrd1 and mammalian gp78 E3 ligases that participate in ER-associated degradation (ERAD), a mechanism of ER protein quality control (Smith et al., 2011). In detail, the Dsc complex contains a RING domain containing protein Dsc1, a ubiquitin-associated (UBA) domain-containing rhomboid pseudoprotease Dsc2, as well as a UBX-domain containing protein Dsc5 (Stewart et al., 2012; Stewart et al., 2011). These structural domains are analogous in their subunit

architecture to the budding yeast ERAD components Hrd1, Der1 and Ubx2 respectively (Bays et al., 2001), and the mammalian ERAD components gp78, UBAC2 and UBXD8 respectively (Christianson et al., 2012).

In ERAD, transmembrane multi-subunit E3 ligases recognize and ubiquitinate misfolded or mutant proteins in the ER, which are then targeted for degradation by the proteasome. Subsequently, the AAA-ATPase Cdc48 or its mammalian counterpart p97 is then responsible for subsequent membrane protein extraction and degradation (Stolz et al., 2011). Importantly, in both budding and fission yeast, aggravating genetic interactions exist between Dsc E3 ligase and multivesicular body pathway components, suggesting that Dsc E3 ligase may participate in post-ER protein degradation/processing (Henne et al., 2011; Stewart et al., 2011). Finally, the Dsc E3 ligase is also conserved in budding yeast. Tul1 is the homolog for Dsc1 in *S. pombe*, and has been shown to be responsible for recognition of a mutant carboxypeptidase that has a charged residue in the transmembrane segment and its sorting into multivesicular bodies (Reggiori and Pelham, 2002). SREBPs are absent in budding yeast (Figure 1.3), further arguing for a post-ER protein quality control function of Dsc E3 ligase beyond SREBP cleavage.

SREBP pathway and fungal pathogenesis

Fungal pathogens remain detrimental to human health and agriculture. To date, fungal SREBPs have also been characterized in the human pathogens *Cryptococcus neoformans* and *Aspergillus fumigatus* (Chang et al., 2007; Chun et al., 2007; Hughes et al., 2005; Lee et al., 2007; Osborne and Espenshade, 2009; Willger et al., 2008). *Aspergillus fumigatus* is a major cause of life-threatening infections in

immunocompromised individuals (Latge, 1999). Notably, *Aspergillus fumigatus* SREBP homolog SrbA as well as Dsc E3 ligase homologs are required for pathogenesis (Willger et al., 2012; Willger et al., 2008). Our group has also revealed *C. neoformans* Sre1 as required for regulating ergosterol biosynthesis (Chang et al., 2007), as well as a role for *C. neoformans* Site-2 protease for *C. neoformans* Sre1 cleavage and subsequent fungal pathogenesis (Bien et al., 2010). In addition, we have recently identified a putative SREBP homolog called Cph2 in the ascomycete fungal pathogen *Candida albicans*, which is required for yeast-to-hypha growth transition and virulence (Lane et al., 2001a; Lane et al., 2001b). Collectively, these studies revealed that fungal SREBPs are transcription factors required for fungal pathogenesis (Bien and Espenshade, 2010). In terms of plant pathogens, the fungal rice blast pathogen *Magnaporthe oryzae*, which causes destructive rice diseases and crop losses worldwide (Wilson and Talbot, 2009), also contains a putative SREBP homolog. Because many other pathogenic fungi contain a SREBP pathway, study of the molecular underpinnings of SREBP cleavage activation may provide a mechanistic basis for antifungal drug design.

Thesis aims

My work in the Espenshade lab followed the discovery of the Dsc E3 ligase complex as genetically required for fission yeast SREBP cleavage activation. At this stage, the mechanism of how the Dsc E3 ligase mediates cleavage and the responsible fungal protease remains unknown. In my thesis work, I sought to establish Sre2 as a model to understand the yeast SREBP cleavage mechanism using a two-pronged approach: (1) from the substrate perspective, I defined the structural requirements for fission yeast SREBP cleavage; (2) I monitored the post-cleavage fate of the SREBP C-terminus to define the mechanism of SREBP processing.

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Figure Legends

Figure 1.1 The mammalian SREBP pathway.

When sterol levels are high, the SREBP-Scap complex is retained in the endoplasmic reticulum (ER) by binding to INSIG. Under sterol deplete conditions, SREBP-Scap is transported to the Golgi apparatus where SREBP is sequentially cleaved by the Golgi Site-1 protease (S1P) and Site-2 protease (S2P). Soluble SREBP N-terminus then enters the nucleus and activates genes required for lipid synthesis and uptake.

Figure 1.2 *Schizosaccharomyces pombe* homologs of mammalian SREBP.

Transmembrane helix prediction plots of human SREBP-1a and *Schizosaccharmyces pombe* homologs Sre1 and Sre2. These predictions are generated using the TM-HMM 2.0 software. Heavy bars denote regions of high sequence identity. The amino acid position of the highly conserved tyrosine in the basic helix-loop-helix zipper domain (bHLH-zip) is indicated (Adapted from Hughes et al., 2005).

Figure 1.3 SREBP conservation from fungi to mammals.

Species for which SREBPs are conserved are indicated in blue. SREBPs are conserved from fungi to animals, but not in bacteria, plants or archaea kingdom. SREBP is conserved in both Basidiomycota and Ascomycota, the two major fungal phyla. Representative species are indicated for each phylum. Note that under the Ascomycota phylum, SREBP is conserved in the fission yeast *Schizosaccharomyces pombe* but not the budding yeast *Saccharomyces cerevisiae*.

Figure 1.4 SREBP and Site-2 protease conservation from fungi to mammals.

Species for which SREBPs are conserved are indicated in blue, while species for which both SREBP and Site-2 protease are conserved are indicated in red. Note that Site-2 protease is only conserved in the basidiomycete fungi *Cryptococcus neoformans* and *Cropinopsis cinerea*, but not in the ascomycete fungi *Schizosaccharomyces pombe* or *Aspergillus fumigatus*.

Figure 1.5 Sre1 pathway in *Schizosaccharomyces pombe*.

Under low oxygen conditions, the *S. pombe* SREBP Sre1 is transported from endoplasmic reticulum (ER) to the Golgi apparatus, where it is proteolytically activated by the Dsc E3 ligase complex Dsc1 through Dsc5, E2 ubiquitin conjugating enzyme Ubc4 and the AAA-ATPase Cdc48. The soluble N-terminus of Sre1 enters the nucleus and activates genes required for adaptation to hypoxia.

Figure 1.1

Mammalian SREBP

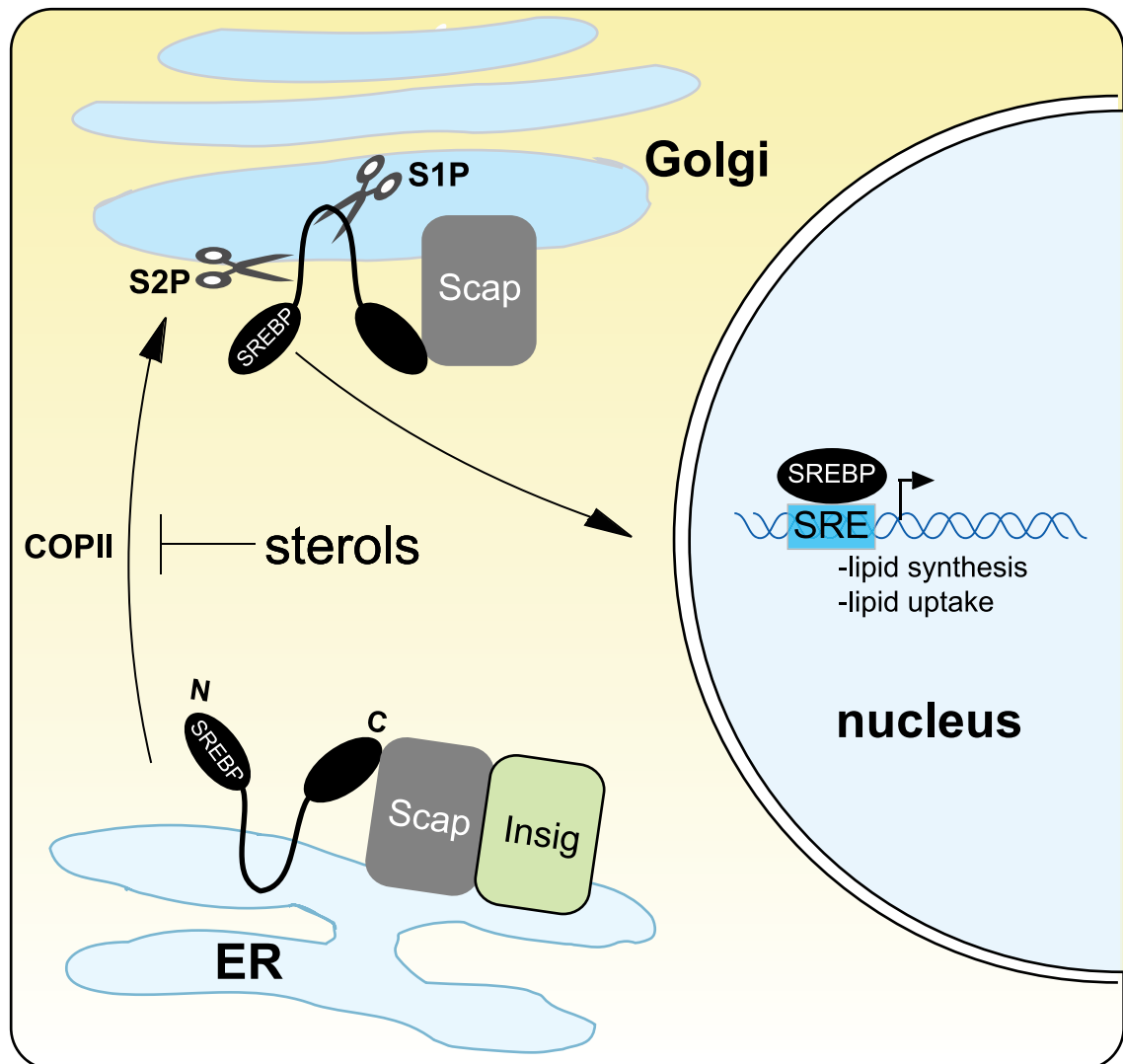


Figure 1.2

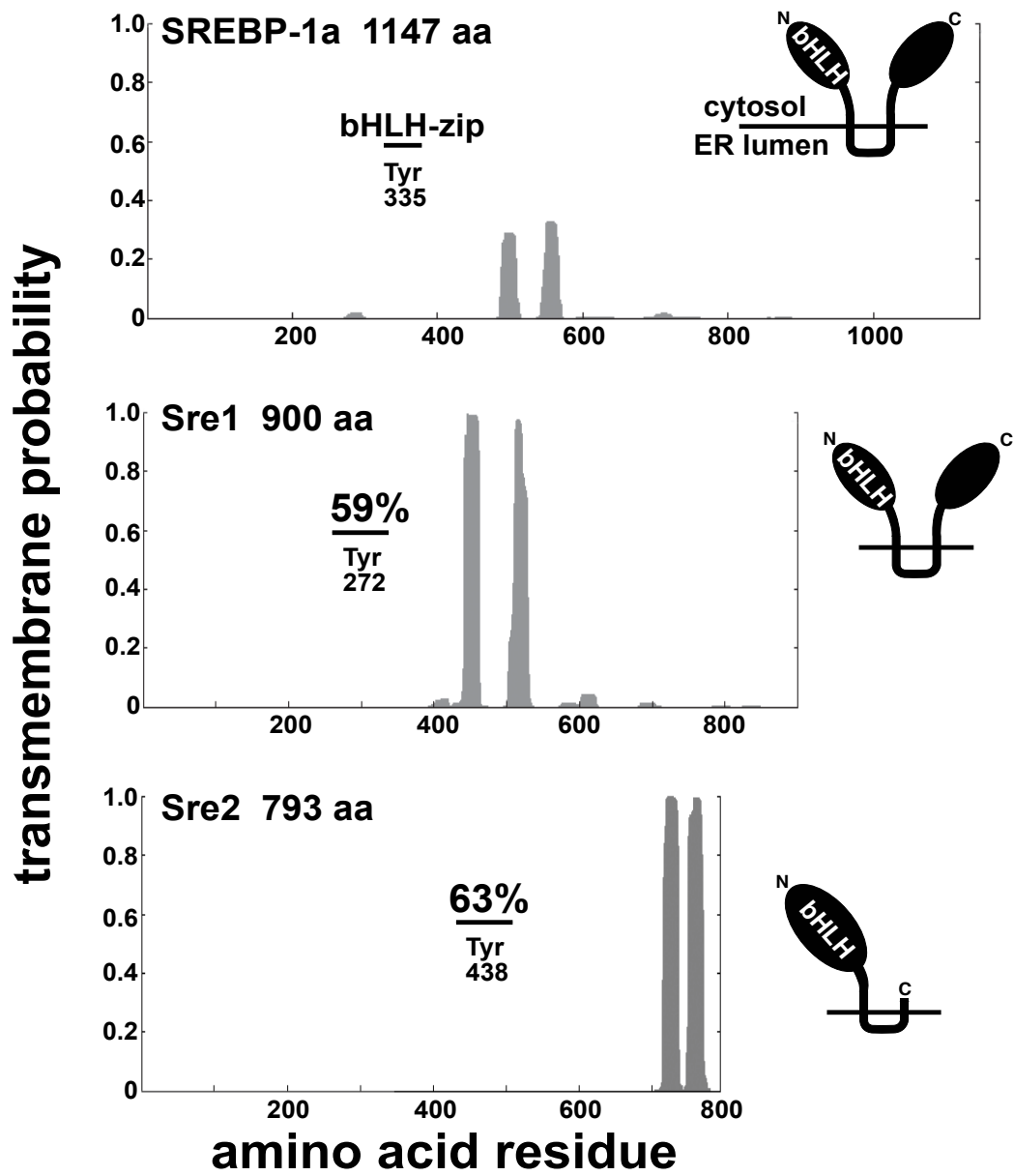


Figure 1.3

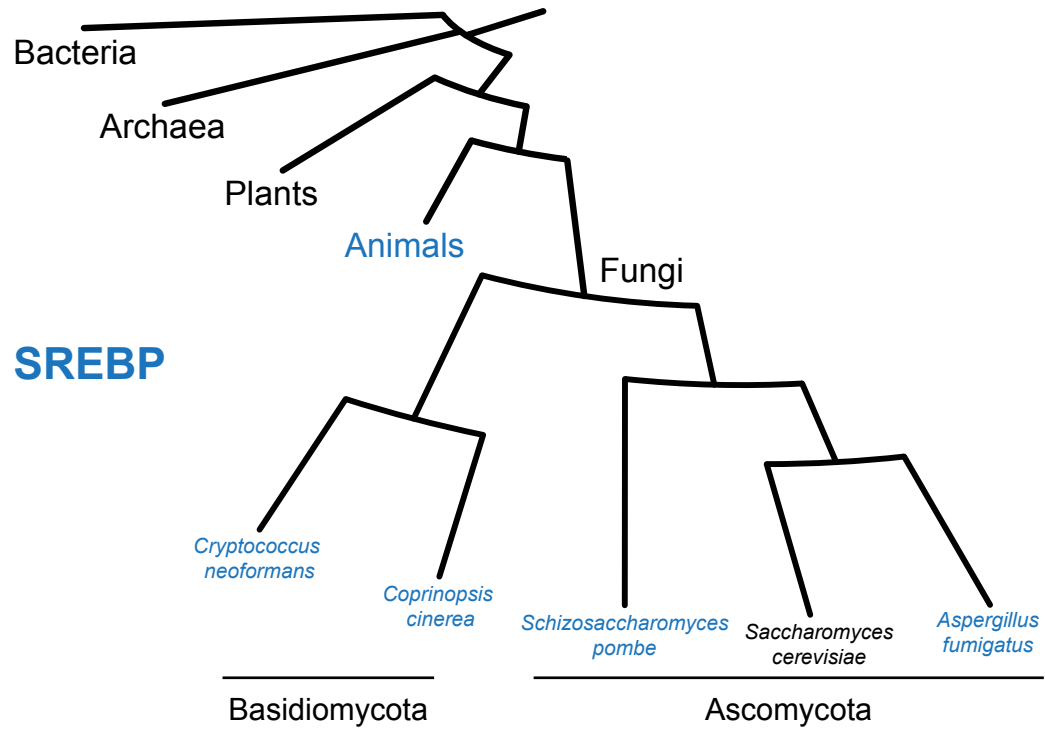


Figure 1.4

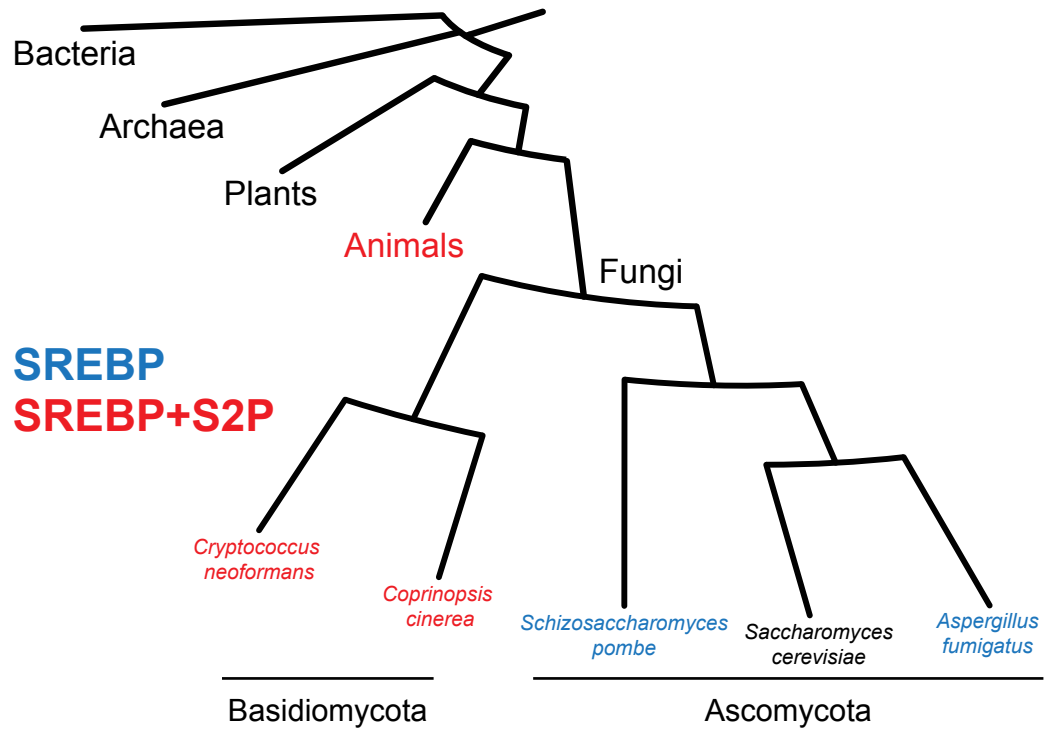
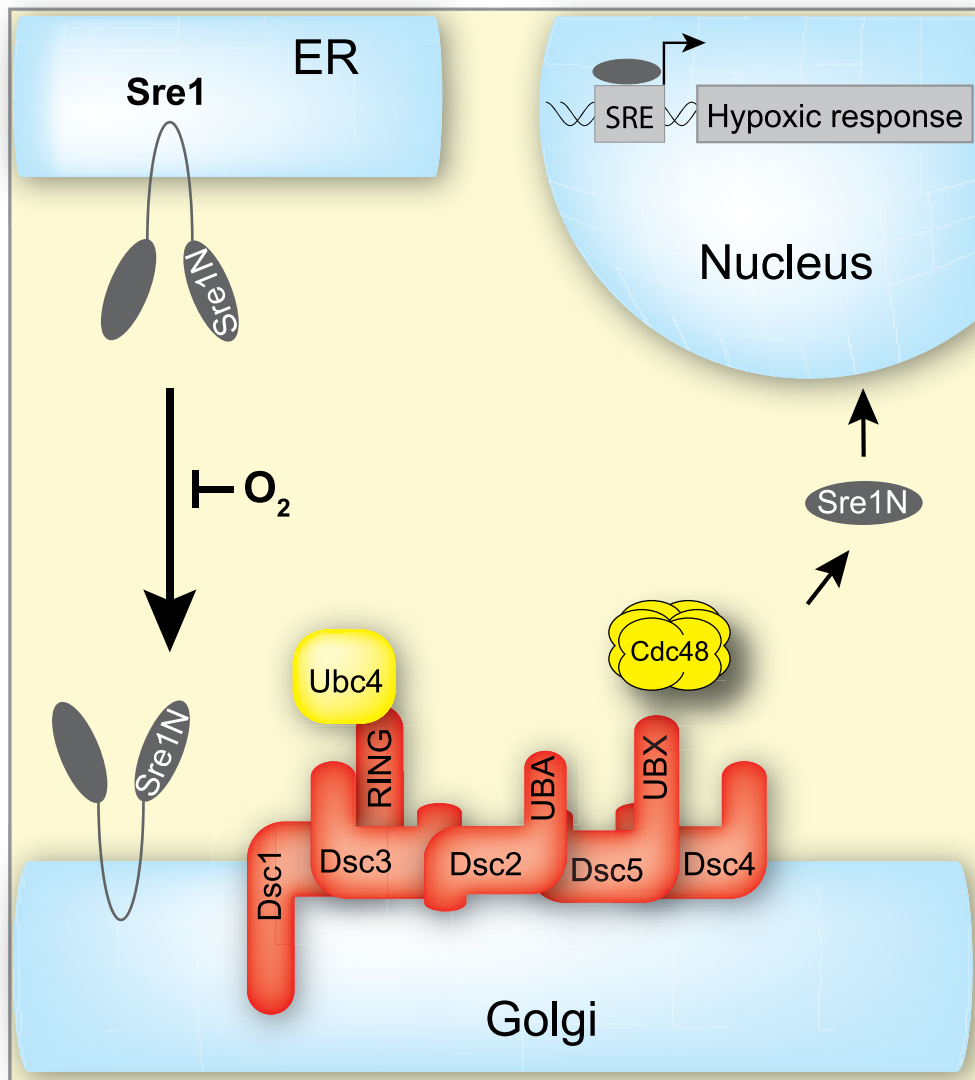


Figure 1.5



Chapter 2

Structural Requirements for Sterol Regulatory Element-Binding Protein (SREBP) Cleavage in Fission Yeast

This chapter is an edited version of the manuscript, “Structural Requirements for Sterol Regulatory Element-Binding Protein (SREBP) Cleavage in Fission Yeast” by R. Cheung and P. J. Espenshade, published in the Journal of Biological Chemistry, Volume 288, Issue 28, 20351-20360. © 2013 American Society for Biochemistry and Molecular Biology, Inc.

Summary

Sterol regulatory element-binding proteins (SREBPs) are central regulators of cellular lipid synthesis and homeostasis. Mammalian SREBPs are proteolytically activated and liberated from the membrane by Golgi Site-1 and Site-2 proteases. Fission yeast SREBPs, Sre1 and Sre2, employ a different mechanism that genetically requires the Golgi Dsc E3 ligase complex for cleavage activation. Here, we established Sre2 as a model to define structural requirements for SREBP cleavage. We showed that Sre2 cleavage does not require the N-terminal basic helix-loop-helix zipper transcription factor domain (bHLH-zip), thus separating cleavage of Sre2 from its transcription factor function. From a mutagenesis screen of 94 C-terminal residues of Sre2, we isolated 15 residues required for cleavage and further identified a glycine-leucine sequence required for Sre2 cleavage. Importantly, this sequence is located at a conserved distance before the first transmembrane segment of both Sre1 and Sre2, and cleavage occurs between this sequence and the membrane. Bioinformatic analysis revealed a broad conservation of this novel glycine-leucine motif in SREBP homologs of ascomycete fungi, including the opportunistic human pathogen *Aspergillus fumigatus* where SREBP is required for virulence. Consistent with this, the sequence was also required for cleavage of the oxygen-responsive transcription factor Sre1 and adaptation to hypoxia, demonstrating functional conservation of this cleavage recognition motif. These cleavage mutants will aid identification of the fungal SREBP protease and facilitate functional dissection of the Dsc E3 ligase required for SREBP activation and fungal pathogenesis.

Introduction

Cellular lipid synthesis and homeostasis are centrally regulated by sterol regulatory element-binding protein (SREBP) transcription factors (Espenshade and Hughes, 2007; Shao and Espenshade, 2012). These membrane-bound transcription factors contain two transmembrane segments and are inserted into the endoplasmic reticulum (ER) in a hairpin orientation with the N- and C-termini projecting into the cytosol (Espenshade and Hughes, 2007). SREBP N-terminus contains a basic-helix-loop-helix zipper (bHLH-zip) transcription factor domain that is required for DNA binding and transcriptional activation of its target genes required for cholesterol and fatty acid synthesis (Espenshade and Hughes, 2007). In mammalian cells following sterol-regulated transport to the Golgi, SREBPs are activated through sequential cleavage by the Golgi-resident Site-1 protease and Site-2 protease. Specifically, Site-1 protease is a subtilisin/kexin-like, serine protease that cleaves in the SREBP luminal loop, splitting the molecule in half. This first cleavage event enables the Site-2 protease zinc metalloprotease to cleave within the first transmembrane segment (TM1) and release the soluble SREBP N-terminal transcription factor from the membrane (Rawson et al., 1997). In mammals, S2P is essential for membrane release and SREBP activity.

Fungal SREBPs have been characterized in *Schizosaccharomyces pombe* and the human pathogens *Cryptococcus neoformans* and *Aspergillus fumigatus* (Chang et al., 2007; Chun et al., 2007; Hughes et al., 2005; Lee et al., 2007; Osborne and Espenshade, 2009; Willger et al., 2008). Collectively, these studies revealed that fungal SREBPs are oxygen-responsive transcription factors required for adaptation to hypoxia and fungal pathogenesis (Bien and Espenshade, 2010). Fission yeast *Schizosaccharomyces pombe*

contains two SREBP homologs: Sre1 and Sre2 (Hughes et al., 2005). Sre1 is an oxygen-responsive transcription factor that is cleaved under low oxygen to promote adaptation to hypoxia (Hughes et al., 2005). In contrast, cleavage of the less well-characterized Sre2 occurs in the presence of oxygen and is unregulated. This feature of Sre2 makes it a useful model to study structural requirements for cleavage under routine cell culture conditions rather than low oxygen (Stewart et al., 2012; Stewart et al., 2011).

Fission yeast lacks an identifiable Site-2 protease homolog, suggesting a different mechanism for SREBP cleavage (Espenshade and Hughes, 2007). Previously, we showed that Sre1 is cleaved at a cytosolic position, instead of within TM1, consistent with Site-2 protease-independent cleavage. Genetic evidence revealed that both Sre1 and Sre2 cleavage requires a multi-subunit Golgi-localized E3 ligase, which we named Dsc (defective for SREBP cleavage) (Stewart et al., 2012; Stewart et al., 2011). The Dsc E3 ligase, composed of Dsc1 through Dsc5, is a stable membrane complex containing the RING-domain E3 ubiquitin ligase Dsc1 (Stewart et al., 2012). Consistent with its role in SREBP activation, *Aspergillus fumigatus* Dsc E3 ligase is also required for fungal virulence (Willger et al., 2012).

Despite genetic evidence of cleavage requirements for fission yeast SREBPs, the mechanism of how the Dsc E3 ligase mediates cleavage and the responsible fungal protease remains unknown. To aid uncoupling mechanistic steps of yeast SREBP cleavage, we dissected the cleavage mechanism from a substrate perspective. In the present study, we defined the structural requirements for Sre2 cleavage using serial truncation analysis and a site-directed mutagenesis screen. We isolated 15 single amino acid mutations in Sre2 that prevent cleavage. In addition, we identified a conserved, 7-

amino acid glycine-leucine sequence in Sre2 required for cleavage. Importantly, the glycine-leucine sequence was required for Sre1 cleavage, confirming the function of this conserved cleavage recognition motif. This motif is broadly conserved across ascomycete fungi, whose members lack homologs of the intramembrane-cleaving Site-2 protease present in mammals. These findings demonstrate a role for this conserved glycine-leucine motif in fungal SREBP cleavage activation, provide tools for dissecting the mechanism of SREBP cleavage, and identify the SREBP C-terminus as a target for antifungal therapy for pathogenic fungi that contain a relevant conserved SREBP pathway (Blatzer et al., 2011; Chang et al., 2007; Chang et al., 2009; Chun et al., 2007; Willger et al., 2008).

Results

Minimal sequence required for Sre2 cleavage

Sre2 is a 793 a.a. protein with two transmembrane segments (a.a. 714-737 and a.a. 749-771) (Figure 2.1A). To determine what region of Sre2 is required for Dsc-dependent cleavage, we generated serial N-terminal truncations of Sre2 and assayed cleavage of these mutants in whole cell extracts by immunoblotting (Figure 2.1A). To monitor Sre2 cleavage events, we inserted a 3xFLAG epitope at the N-terminus of Sre2 truncations, thereby allowing us to distinguish Sre2 precursor from Sre2 N-terminal cleaved form. Deletion of the N-terminal half of Sre2, a.a. 1-422, had no effect on cleavage. N-terminal truncated Sre2 (a.a. 423-793) showed a cleavage pattern similar to full-length Sre2 (Stewart et al., 2011), with the majority of Sre2 in the cleaved form at steady state (Figure 2.1B, *lane 1*). Importantly, this cleavage required Dsc1 (Figure 2.1B, *lane 2*), the Golgi E3 ligase required for the cleavage activation of both Sre1 and Sre2 precursors (Stewart et al., 2011). This result indicated that cleavage does not require Sre2 a.a. 1-422. Further Sre2 truncations across the bHLH-zip domain (a.a. 426-516) demonstrated that this domain is also not required for cleavage (Figure 2.1B, *lanes 3 and 5*), despite the requirement of this domain for DNA binding and transcription factor activity. Thus, the functions of Sre2 as a transcription factor and a substrate for Dsc-dependent cleavage are separable. Finally, Sre2 failed to cleave when we truncated Sre2 to a.a. 588 and the Sre2 precursor migrated at the same position in the presence or absence of *dsc1* (Figure 2.1B, *lanes 7 and 8*). Taken together, these results suggest that a.a. 1-522 including the bHLH-zip domain of Sre2 are not required for cleavage. However, sequences in the C-terminal portion of Sre2 are important for cleavage.

Cleavage of Sre2 occurs in the cytosol

Dsc-dependent cleavage of the oxygen-regulated Sre1 transcription factor occurs in the cytosol at a position ~10 amino acids before the first transmembrane segment (Stewart et al., 2011). Next, we mapped the cleavage site for Sre2. Because Sre2 cleavage occurs at a site close to its C-terminus, Sre2 precursor and its cleaved nuclear form are not readily resolved by SDS-PAGE. Therefore, we chose to use an Sre2 model substrate, Sre2 a.a. 423-793 (hereafter, Sre2-MS), that is efficiently cleaved in a Dsc-dependent manner to analyze Sre2 sequences required for cleavage (Figure 2.1B, *lanes 1-2*). We verified that the N-terminus of GFP-Sre2 a.a. 423-793 translocates to the nucleus, demonstrating that Sre2-MS is functionally imported after Dsc-dependent cleavage (data not shown). These data are consistent with studies on mammalian SREBP-2 showing that the bHLH-zip domain functions as a nuclear import signal (Lee et al., 2003).

Cleavage of mammalian SREBPs by S2P occurs within the first transmembrane segment between a leucine and cysteine residue, both of which are not present in Sre2 (Duncan et al., 1997). To estimate the position of Sre2 cleavage, we generated two C-terminal truncations of Sre2-MS encoding a.a. 423-697 and 423-712. These two size standards truncated Sre2-MS at cytosolic positions prior to the first transmembrane segment (Figure 2.1A). Cleaved Sre2-MS migrated between the two C-terminal truncations (Figure 2.2A, *lane 4*). Thus, Sre2 is cleaved between a.a. 697 and 712 in the cytosol, consistent with cytosolic cleavage of Sre1 (Stewart et al., 2011). Notably, the cytosolic cleavage sites of Sre1 and Sre2 are equidistant (~10 amino acids) from their respective first transmembrane segments. These results reinforce the observation that SREBP cleavage in *S. pombe* is mechanistically distinct from that in mammals, whereby

cleavage occurs within the first transmembrane segment.

Cleavage of Sre2 model substrate requires Dsc E3 ligase complex, Cdc48 and E2 enzyme Ubc4

Cleavage of Sre1 and Sre2 requires each of the subunits of the Golgi Dsc E3 ligase complex Dsc1 through Dsc5, AAA-ATPase Cdc48 and the E2 ubiquitin-conjugating enzyme Ubc4 (Stewart et al., 2012; Stewart et al., 2011). To investigate whether cleavage requirements of Sre2-MS parallel those of Sre1 and Sre2, we assayed cleavage of Sre2-MS in cells lacking each of these components. We found that Dsc1 through Dsc4 were strictly required for Sre2-MS cleavage (Figure 2.2B, *lanes 2-5*). In *dsc5Δ* cells, a small amount of Sre2-MS cleaved form was detectable (Figure 2.2B, *lane 6*), consistent with partial requirement of *dsc5* for cleavage of full length Sre2 precursor (Stewart et al., 2012). *cdc48-4* contains a mutation (E325K) that lies in the Walker B motif in the AAA-ATPase domain of Cdc48 (Stewart et al., 2012). This mutation causes a complete block of Sre2 cleavage compared to other *cdc48* mutant alleles identified in a previous genetic screen, such as *cdc48-2* (A586V) and *cdc48-3* (E731K) in the D2 domain of Cdc48, that cause a severe impairment but not complete block (Stewart et al., 2012). Consistent with this, Sre2-MS cleavage was blocked completely in *cdc48-4* (Figure 2.2B, *lane 7*) but not in *cdc48-2* and *cdc48-3* (data not shown). Finally, a temperature-sensitive allele *ubc4-P61S* of the essential E2 enzyme Ubc4 was utilized to test its role in Sre2-MS cleavage. Upon shifting to non-permissive temperature to block Ubc4 function, the precursor form of Sre2-MS accumulated in *ubc4-P61S*, but not wild-type cells (Figure 2.2C). This result indicated that Sre2-MS cleavage requires Ubc4.

Taken together, these results demonstrate that Sre2-MS shares all functional requirements for Dsc-dependent cleavage with full-length Sre2 (a.a. 1-793) and Sre1 (Stewart et al., 2012; Stewart et al., 2011), and thus is a model substrate for further mutational studies.

Site-directed mutagenesis screen of Sre2 model substrate

To understand comprehensively the requirements for Sre2 cleavage, we set out to screen for cleavage defects by site-directed scanning mutagenesis of Sre2-MS. Based on protein secondary structure prediction programs for structured regions, and given that cleavage of both Sre1 and Sre2 occurs close to the first transmembrane segment, we focused our studies on the C-terminal 117 amino acids of Sre2-MS, mutating residues 677-793 (Figure 2.3A). First, we tested a total of 94 amino acid residues by mutating amino acids either in single, double or triple mutants (Table 2.S1). We chose not to mutate hydrophobic residues in the transmembrane segments as these would likely not be well expressed. Mutants were grouped into three classes according to cleavage efficiency [the ratio of processed form (N) to precursor form (P)] (Table 2.1). 51 mutants showed normal cleavage ($N > P$), 28 showed a defect in cleavage ($N \approx P$), and 15 showed a robust block in cleavage ($P > N$). Examples of each class are shown in Figure 2.3B. For double or triple mutants that demonstrated a robust cleavage block ($P > N$), we further tested cleavage requirements by mutating individual amino acids to alanine for cytosolic residues and to leucine for residues within the transmembrane segments. For selective residues that are structurally similar to alanine or leucine, we mutated amino acids to charged residues such as glutamate or threonine. Figure 2.3A and Table 2.1 summarize the results of the Sre2-MS mutagenesis screen.

From the screen, we identified a short, 7-amino-acid glycine-leucine region required for Sre2 cleavage (a.a. 678-684) (Figure 2.3A). Interestingly, this glycine-leucine stretch is present in Sre1 (Figure 2.4A) and is roughly equidistant from the first transmembrane segment in both Sre1 and Sre2 (27 and 29 amino acids, respectively). These observations suggested a role for the glycine-leucine region in Sre2 cleavage. To investigate whether this conserved sequence (a.a. 678-684) was required for cleavage, we assayed cleavage of single residue mutants. Notably, mutating 5 out of 7 residues blocked cleavage when tested individually (Figure 2.4B, *lanes 3-7*).

In addition, we identified 10 other single residues required for cleavage (Figure 2.3A). Two residues, M704 and S705, are located ~10 amino acids before the first transmembrane segment close to the cleavage site (Figure 2.4C, *lanes 3-12*). We identified only a single residue, K743, required for cleavage in the short endoplasmic reticulum lumenal loop of Sre2. Mutation of 5 residues in the second transmembrane segment blocked cleavage (E755, S765, P767, D770, W771) while none in the first transmembrane segment were absolutely required (Figure 2.3A). Finally, two charged residues in the cytosolic Sre2 C-terminal tail (R778 and E788) were also required for cleavage. In total, 15 C-terminal residues were found to be essential for Sre2-MS cleavage (Figure 2.3A).

Validation of sequence requirements in full-length Sre2

To independently test and verify the cleavage requirement for residues identified in the Sre2-MS screen, we generated each of the cleavage mutants in full-length Sre2-GFP. Fusion of GFP to the C-terminus allows clear discrimination between the precursor

(P) and cleaved nuclear (N) forms of Sre2 (Stewart et al., 2011). Wild-type Sre2-GFP was cleaved constitutively to generate Sre2N and cleavage was blocked in *dsc1Δ* cells (Figure 2.5A, *lanes 1-2*). Mutation of each of the five glycine-leucine region residues blocked cleavage of full-length Sre2-GFP (Figure 2.5A, *lanes 3-7*), emphasizing the importance of this sequence. Indeed, we observed a complete cleavage block for each of the additional 10 residues identified using Sre2-MS, examples of which are shown in Figure 2.5B. These results validate Sre2 a.a. 423-793 as a model substrate and confirm the importance of these 15 residues for cleavage of full-length Sre2.

As a complementary approach to test the cleavage requirements for individual Sre2 residues, we assayed localization of different GFP-Sre2 fusion proteins in cells lacking endogenous Sre2. GFP fused to the N-terminus of wild-type Sre2 translocated to the nucleus after its release from the membrane (left panel of Figure 2.5C). Failure to cleave GFP-Sre2 in *dsc1Δ* cells caused GFP-Sre2 to localize in punctate structures (middle panel of Figure 2.5C) (Stewart et al., 2011). Consistent with a requirement for Dsc-dependent cleavage, GFP-Sre2 G683 containing a mutation in the glycine-leucine stretch also localized to punctate structures (right panel of Figure 2.5C). We tested all of the 15 individual cleavage mutants in this GFP-Sre2 assay and each failed to show nuclear localization equivalent to wild-type GFP-Sre2 (data not shown). Combined with the full-length Sre2-GFP cleavage assays, these data demonstrate that full-length Sre2 cleavage requires each of the 15 residues isolated using Sre2-MS, showing the physiological importance of these sequences.

Bioinformatic analysis reveals wide conservation of glycine-leucine motif in ascomycete fungi

Our mutagenesis screen identified a glycine-leucine sequence in Sre2 (a.a. 678-684) that is required for cleavage and conserved in Sre1 (Figure 2.4A). Interestingly, the distance of this sequence from the first transmembrane segment (~30 amino acids) is also conserved between Sre2 and Sre1. To investigate whether the glycine-leucine region is conserved beyond *S. pombe*, we searched for glycine-leucine sequences in SREBPs ranging from fungi to mammals using a defined set of bioinformatic criteria. Given the short length of the conserved sequence, parameter definition was critical for the motif search and subsequent consensus motif generation. First, we isolated SREBPs from all species by sequence similarity search. We defined SREBPs by the presence of a specific tyrosine residue in the first helix of bHLH transcription factors. For each SREBP, we predicted coordinates for transmembrane segments using transmembrane prediction software, and we used only SREBPs that contained a predicted TM for subsequent analysis. Given that the glycine-leucine sequence is located at a conserved distance from TM1s of Sre1 and Sre2, we selected a 50-amino-acid stretch that is centered 55 amino acids from the beginning of TM1 for each SREBP. We then performed a sequence alignment using all isolated 50-amino-acid sequences. In this way, we identified a consensus sequence corresponding to the glycine-leucine motif (Figure 2.6A). We identified the glycine-leucine motif in all ascomycete fungi analyzed, including pathogenic fungi *Aspergillus fumigatus* and *Magnaporthe oryzae*, but not basidiomycete fungi, like *Cryptococcus neoformans* (Table 2.S2). Interestingly, *C. neoformans* utilizes S2P to cleave and activate SREBP like mammalian cells (Bien et al., 2009). Collectively,

this bioinformatic analysis identified a conserved glycine-leucine motif present in ascomycete fungi that lack S2P, further supporting a role for this sequence in SREBP cleavage.

Sre1 cleavage requires glycine-leucine motif

Having identified this conserved glycine-leucine motif, we next tested whether motif function is conserved between SREBPs. Fission yeast strains were generated that expressed wild-type or *sre1* glycine-leucine motif mutants at the heterologous *his3* locus (Burke and Gould, 1994). We cultured cells under low oxygen and assayed Sre1 cleavage by immunoblotting. Wild-type Sre1 precursor was cleaved to generate active Sre1N under hypoxia (upper, middle and lower panels of Figure 2.6B, *lanes 1-4*). Induction of Sre1 expressed from the *his3* locus was reduced compared to wild-type cells, perhaps due to the absence of DNA elements required for key positive feedback regulation (Hughes et al., 2005; Lee et al., 2011; Porter et al., 2012). As a control, we tested mutation of Sre1 N408E in the 7-amino-acid conserved motif, corresponding to the second position in the motif that was not required for cleavage of Sre2 (a.a. 679) (Figure 2.6A). In agreement, mutation of this residue did not affect cleavage of Sre1 (upper panel of Figure 2.6B, *lanes 5-8*). Then, we tested 4 of 5 required residues in the motif for Sre1 cleavage. Notably, mutation of glycine at the third position (G409), methionine at the fourth position (M410), glycine at the sixth position (G412) and leucine at the seventh position (G413) blocked Sre1 cleavage under low oxygen (middle and lower panels of Figure 2.6B, *lanes 5-12*). These results were consistent with Sre2-MS, showing cleavage defects at the third, fourth, sixth and seventh positions of the motif (Sre2 a.a. 678, 680, 683 and 684) (Figure

2.6A). As expected, deletion of the E3 ligase *dsc1* abolished Sre1 cleavage (middle panel of Figure 2.6B, *lane 13*). In these experiments, the cleavage machinery functioned normally inasmuch as endogenous Sre2 cleavage was normal (data not shown).

As an independent test, we assayed the ability of these *sre1* mutants to grow in the presence of cobalt chloride, a hypoxia mimetic (Lee et al., 2007; Stewart et al., 2011). Wild-type cells, but not *sre1Δ* cells, grow under low oxygen (Hughes et al., 2005). If Sre1 mutants are cleavage-defective, we expect to observe reduced growth on cobalt chloride, reminiscent of growth defects in *sre1Δ* and *dsc1Δ* cells. Consistent with results from the cleavage assays in Figure 2.6B, Sre1 mutants blocked for cleavage failed to support wild-type growth on cobalt chloride (Figure 2.6C), and growth defects correlated with the severity of observed Sre1 cleavage defects (Figure 2.6B). These results demonstrate that the glycine-leucine motif is required for Sre1 cleavage, and together with the bioinformatic data suggest that this motif has a conserved function in SREBP cleavage in ascomycete fungi.

Discussion

Genetic studies revealed requirements for *dsc1* through *dsc6* in the cleavage activation of the yeast SREBP transcription factors Sre1 and Sre2 (Stewart et al., 2012; Stewart et al., 2011). Dsc1 through Dsc5 constitute the Dsc E3 ligase, a stable Golgi membrane complex containing the RING-domain E3 ubiquitin ligase Dsc1 (Stewart et al., 2012). *dsc6* codes for Cdc48, an AAA-ATPase that binds to the Dsc E3 ligase through the ubiquitin regulatory X (UBX) domain of Dsc5 (Stewart et al., 2012). Previous studies indicate that the mechanism of cleavage is the same for Sre1 and Sre2 (Stewart et al., 2012; Stewart et al., 2011), except that ER exit of Sre1 is regulated by sterols and oxygen and Sre2 exits the ER constitutively (Hughes et al., 2005; Porter et al., 2012). Our working model for yeast SREBP (Sre1 and Sre2) cleavage is as follows: (1) SREBP moves from the ER to Golgi; (2) SREBP binds to the Dsc E3 ligase; (3) SREBP is ubiquitinated by the E2 ubiquitin-conjugating enzyme Ubc4 and E3 ligase Dsc1; (4) SREBP is subsequently cleaved by an unidentified protease releasing the N-terminal transcription factor domain from the membrane.

To develop tools that will allow us to test this model and dissect the mechanism of SREBP cleavage, we investigated the structural requirements for SREBP cleavage. We focused initially on Sre2 because its cleavage is constitutive, allowing us to study sequences required for cleavage under routine cell culture conditions, rather than having to induce cleavage under hypoxia. Truncation analysis revealed that Sre2 cleavage does not require its bHLH-zip domain and demonstrated that a minimal substrate of 271 amino acids is still cleaved (Figure 1). This result indicates that the functions of Sre2 as a transcription factor and a substrate for Dsc-dependent cleavage are separable. Using a

truncated Sre2 model substrate, we identified 15 residues required for cleavage of full-length Sre2 and uncovered a novel SREBP cleavage motif. We discuss these mutants in light of our current understanding of SREBP cleavage in *S. pombe* and mammals. Mutants blocked for Sre2 cleavage could be defective in any of the 4 steps outlined in our working model for SREBP cleavage. Sre2 cytosolic mutations could disrupt binding to COPII proteins required for sorting into vesicles and ER exit (Barlowe et al., 1994). However, none of the 15 Sre2 mutants localized to the ER when tested in either the GFP-Sre2 (Figure 2.5C) or GFP-Sre2-MS localization studies (data not shown), suggesting that ER exit is normal in the mutants. In addition, Sre2 mutants showed wild-type expression (Figure 4B and 2.4C), suggesting that the proteins are properly folded and unlikely to be substrates for ER retention and endoplasmic-reticulum associated degradation (Hughes et al., 2009). Determinants required for ER exit may be located between a.a. 523-676, a region required for cleavage (Figure 1B), but not subjected to site-directed mutagenesis.

Function of conserved glycine-leucine motif in SREBP cleavage

Several lines of evidence suggest that the glycine-leucine motif is a key determinant for Dsc-dependent SREBP cleavage. First, the glycine-leucine motif is located at a conserved distance from the first transmembrane segment of Sre1 and Sre2 in *S. pombe* and more than 20 other ascomycete SREBPs. Second, the presence of the glycine-leucine motif correlates with the lack of Site-2 protease homologs, i.e. every ascomycete fungus that contains the conserved glycine-leucine motif lacks a Site-2 protease homolog (Bien and Espenshade, 2010). Third, the glycine-leucine motif is

functionally required for both Sre1 and Sre2 cleavage in *S. pombe*. Fourth, this motif is broadly conserved among ascomycete fungi.

Protein secondary structure software predicts this novel glycine-leucine motif folds either as alpha helix or beta strand. This motif might represent a hydrophobic interface for interaction with the Dsc E3 ligase or the unidentified fungal SREBP protease. Given that the motif is located at a distance (~20 amino acids) from the cleavage site, it is unlikely to participate in interactions with the protease active site. Indeed, a definitive functional assignment for this motif requires the development of additional assays for Dsc E3 ligase recognition, SREBP ubiquitination, and SREBP proteolytic cleavage.

As noted, the protease that cleaves SREBP in this system is not known. Mammalian SREBP is cleaved sequentially by the Site-1 protease after RXXL in the ER lumenal loop, before it can be cleaved by the Site-2 protease within TM1 to release functional transcription factor domain (Brown and Goldstein, 1999). Site-1 protease belongs to the subtilisin/kexin-like protease family, required for cleaving many proproteins to their active form (Seidah, 2011; Seidah et al., 1998). Interestingly, we have isolated a cleavage mutant in the ER lumenal loop of Sre2 at lysine 743. A charge-conservative mutation from lysine to arginine blocks Sre2 cleavage (Figure 3). Subtilisin/kexin-like proteases typically cleave after lysine or arginine and dibasic sequences (Seidah, 2011; Seidah et al., 1998), as evidenced by normal SREBP cleavage in mammals for the same corresponding K to R mutation (Duncan et al., 1997). Further, in contrast to the cleavage of mammalian SREBP by Site-1 protease at a lumenal RXXL sequence, this sequence is not conserved in Sre2 (Duncan et al., 1997). More generally,

proteases in the subtilisin/kexin-like family cleave after the consensus R-X-(R/K)-R sequence, where X is any amino acid except cysteine (Steiner, 1998). Collectively, these data argue against a Site-1 protease-like cleavage for yeast SREBP.

A Site-2 protease cleavage mechanism is also unlikely given the cytosolic cleavage sites of Sre1 (Stewart et al., 2011) and Sre2 (Figure 2A). Sre2 cleavage defects at methionine 704 and serine 705 represent cleavage mutants close to the cleavage site (Figure 2.3A). These mutants might interfere directly with interaction between Sre2 and the unidentified protease. Consistent with this, the presence of Site-2 protease correlates with cleavage within the membrane (Bien et al., 2009; Bien and Espenshade, 2010), while the absence of Site-2 protease correlates with cytosolic cleavage. Given the lack of Site-2 protease homologs in *S. pombe* and the broader ascomycete fungal phylum, and no detectable cleavage within TM1, a new cleavage mechanism might be employed for ascomycete SREBPs.

Sre2 cleavage mutants are enriched in the second transmembrane segment (TM2) (Figure 2.3A), but a close examination of other SREBP TM2s did not reveal conserved sequences. Interestingly, ubiquitination and sorting of the Pep12 membrane protein into the multivesicular body required insertion of an aspartate residue into its transmembrane segment and Tul1, the *S. cerevisiae* homolog of the Dsc1 E3 ligase (Reggiori and Pelham, 2002). Thus, it is possible that charged residues in a transmembrane segment are a signal for substrate recognition. Sre2 cleavage mutants within TM2 that contain a polar residue, such as glutamate 755, might fail to be recognized by the Dsc1 E3 ligase through the same mechanism.

Identification of the fungal SREBP protease and further characterization of Dsc E3 ligase function will require development of new assays. Importantly, these mutants will aid the identification of the protease and future structure-function studies. For example, genetic suppression of Sre1 cleavage-defective mutants may uncover components required for different steps in the pathway.

Implications for fungal pathogenesis

Ascomycete fungal pathogens remain detrimental to human health and agriculture. For instance, *Aspergillus fumigatus* is a major cause of life-threatening infections in immunocompromised individuals (Latge, 1999). Notably, *Aspergillus fumigatus* SREBP homolog SrbA as well as Dsc E3 ligase homologs are required for pathogenesis (Willger et al., 2012; Willger et al., 2008). Interestingly, the glycine-leucine motif is conserved in *Aspergillus*. In terms of agriculture, the fungal rice blast pathogen *Magnaporthe oryzae*, which causes destructive rice diseases and crop losses worldwide (Wilson and Talbot, 2009), also contains a putative SREBP homolog and the cleavage motif. Thus, this motif may be required for SREBP cleavage activation across ascomycete fungal pathogens. Because many other pathogenic fungi contain an SREBP pathway and the conserved motif, this study provides insight into molecular underpinnings of SREBP cleavage activation that may have broad antifungal applications.

Experimental Procedures

Materials

We obtained yeast extract, peptone and agar from BD Biosciences; Edinburgh minimal medium (EMM) from MP Biomedical; oligonucleotides from Integrated DNA Technologies; alkaline phosphatase from Roche; HRP-conjugated, affinity purified donkey anti-rabbit and anti-mouse IgG from Jackson ImmunoResearch; prestained protein standards from Biorad.

Strains and media

Wild-type haploid *S. pombe* KGY425 and derived strains were grown to log phase at 30°C in YES medium (5 g/liter yeast extract plus 30 g/liter glucose and supplements, 225 mg/liter each of adenine, uracil, leucine, histidine, and lysine) or EMM plus supplements unless otherwise indicated. Yeast transformations were performed as previously described (Hughes et al., 2005). Strains used in this study were derived using standard genetic techniques and are described in Table 2.S3 (Bahler et al., 1998).

Antibodies

We obtained anti-FLAG M2 from Sigma. Antisera to Sre1 (a.a. 1-260) and Sre2 (a.a. 1-426) polyclonal IgG generated against the cytosolic N-terminus of fission yeast Sre1 and Sre2 have been described previously (Hughes et al., 2005).

Plasmids

RCP90 and RCP92 encode truncated, N-terminal fragments of Sre2 (a.a. 423-697

and 423-712) under control of the constitutive cauliflower mosaic virus (CaMV) promoter derived from pSLF101 (Forsburg, 1993). These plasmids were constructed by mutation of the appropriate codons to stop codons using Quikchange II XL mutagenesis. pCaMV-3xFLAG truncation plasmids listed in Figure 1 were generated by truncation of the appropriate amino acids in Sre2 using Quikchange II XL mutagenesis. Sre1 integrating plasmid contains the *sre1*⁺ coding sequence flanked by 800bp of upstream genomic sequence and 500bp of downstream genomic sequence, with a *his3*⁺ marker for chromosomal reconstitution of histidine auxotrophy (Burke and Gould, 1994). Plasmids containing *sre1* wild-type and mutants (pES219, RCP365-RCP369) were linearized with *AscI* and transformed into *sre1*Δ strain, and selected for on minimal medium lacking histidine to generate strains carrying integrated wild-type and mutant alleles of *sre1* at the *his3*⁺ locus.

Low oxygen cell culture

For Sre1 hypoxic cleavage assays, cells growing exponentially in YES medium were collected by centrifugation. Oxygenated medium was removed by aspiration, and cells were resuspended in deoxygenated YES medium under anaerobic conditions inside a Ruskinn Invivo₂ 400 hypoxic workstation (Biotrace, Inc.). Anaerobic conditions were achieved in the workstation using 10% hydrogen gas balanced with nitrogen in the presence of palladium catalyst. YES medium was deoxygenated by preincubation for >24 hr in the hypoxic workstation. After resuspension, cultures were agitated at 30°C, harvested by centrifugation, washed with water and frozen as cell pellets in liquid nitrogen.

Cleavage assays

For Sre1 and Sre2 cleavage assays, whole cell lysates were prepared for immunoblotting analysis. Protein preparation and immunoblotting for *S. pombe* experiments were described previously (Hughes et al., 2005). For Sre1 and Sre2, whole cell lysates were extracted and treated with alkaline phosphatase as described previously (Hughes et al., 2005). For Sre2 model substrate, anti-FLAG M2 antibody was used to detect the precursor (P) and the cleaved N-terminal nuclear form (N). For Sre2-GFP, anti-Sre2 polyclonal antibody was used to detect the precursor (P) and cleaved N-terminal nuclear form (N).

Fluorescence microscopy

A plasmid expressing GFP-*sre2* from the thiamine repressible nmt* promoter (pAH230) was described previously (Stewart et al., 2011). Cleavage mutants were generated in this plasmid using Quikchange II XL mutagenesis. Live cells were imaged on 2% agarose pads using a Zeiss Axioskop microscope equipped with fluorescence and Nomarski optics (Zeiss). Images were captured using a Photometrics Cool Snap EZ CCD camera and IP Lab Spectrum software (Biovision Technologies, Inc.).

Mutagenesis screen of Sre2 model substrate

Sre2 model substrate was expressed from a plasmid containing a truncated form of Sre2 (a.a. 423-793), tagged with a 3xFLAG epitope at its N-terminus, under control of the constitutive CaMV promoter. pCaMV mutant plasmids listed in Table 2.1 and Figure 3 were generated by mutation of the appropriate codons using Quikchange II XL

mutagenesis. In the model substrate, 94 amino acids at the distal end of Sre2 (a.a. 676-793), which spans the conserved glycine-leucine motif and two transmembrane segments, were analyzed for cleavage. These mutants were first mutated in groups of doublets or triplets. For the mutants that demonstrated cleavage defects, individual residues were mutated from each group to analyze the contribution to Sre2 cleavage defect. Cleavage defects in model substrate were confirmed by generating the respective mutants in plasmids expressing full-length *sre2*-GFP under the control of the constitutive CaMV promoter, as well as GFP-*sre2* expressed from the thiamine repressible promoter nmt* (and/or GFP-model substrate fusion protein driven by CaMV promoter). These plasmids were used for examining cleavage and localization, respectively.

Bioinformatics

Conservation of glycine-leucine motif of SREBPs was mined using a custom Perl script. In brief, SREBPs were isolated by protein sequence alignment using BLAST database version 2.2.25 downloaded from National Center for Biotechnology Information (NCBI). For each SREBP, coordinates of each transmembrane segment (TM) were predicted using TM-HMM software version 2.0, executed through a BioPerl module and confirmed with Phobius (Stajich et al., 2002). Only SREBPs that contained one or more predicted TM(s) were used for downstream analysis. To define the consensus glycine-leucine conserved motif, a 50-amino-acid stretch centered 55 amino acids before the first predicted TM was isolated for each SREBP. For all the isolated 50-amino-acid sequences, sequence similarity search was performed to reveal the conserved glycine-leucine motif. A sequence logo was generated using WebLogo 3 (Crooks et al., 2004).

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Author Contributions

R.C. performed all experiments. The manuscript was written and edited by P.J.E. and R.C.

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Figure legends

Figure 2.1. Establishing the minimal Sre2 sequence required for cleavage.

(A) Diagrams for serial truncations of Sre2. bHLH-zipper region (bHLH-zip) and predicted transmembrane domains are indicated by gray and black boxes, respectively.

(B) Serial truncation analysis of Sre2. Western blot probed with anti-FLAG IgG of whole cell lysates. Wild-type or *dsc1Δ* cells carrying FLAG-tagged N-terminal truncations of Sre2 or empty vector (EV) were grown in minimal medium lacking leucine for 16 hours. FLAG epitope is appended at the N-terminus of truncated Sre2 to monitor cleavage. All strains are *sre2Δ*.

Figure 2.2. Cleavage of Sre2 model substrate occurs in the cytosol and requires Dsc E3 ligase complex and E2 enzyme Ubc4.

(A) Western blot probed with anti-FLAG IgG of lysates from *sre2Δ* cells expressing wild-type Sre2 model substrate (MS) (amino acids 423-793) (lane 4), empty vector (EV, lanes 1 or 7), or truncated versions of Sre2 (amino acids 423–697 or 423–712, lanes 2, 3, 5, and 6). (B) Western blot probed with anti-FLAG IgG of lysates from wild-type and indicated deletion or mutant strains. Strains carrying plasmids expressing Sre2-MS were grown in minimal medium lacking leucine for 16 hours. (C) Western blot probed with anti-FLAG IgG of lysates from wild-type and *ubc4-P61S*. Cells were grown to exponential phase at 25°C and shifted to the non-permissive temperature of 36°C for the indicated times. *P* and *N*, precursor and cleaved nuclear forms, respectively. All strains are *sre2Δ*.

Figure 2.3. Mutagenesis screen of Sre2 model substrate reveals sequence requirements for cleavage.

(A) Diagram of predicted membrane topology and mutant classification for Sre2 model substrate (amino acids 423-793). 94 amino acids tested in mutagenesis screen are enclosed in squares. From these residues, mutants showing a robust block in cleavage ($P > N$) are indicated in red, mutants showing cleavage defect ($N \approx P$) are indicated in gray, and residues showing normal cleavage ($N > P$) are indicated in white. Amino acid residues of basic helix-loop-helix zipper domain (bHLH-zip) are enclosed in hexagons. (B) Western blot probed with anti-FLAG IgG of lysates from *sre2Δ* cells expressing the indicated wild-type or mutant Sre2 model substrate. Each mutant defines a category of observed cleavage efficiency. Asterisks denote non-specific cross-reactive bands.

Figure 2.4. Glycine-leucine sequence is required for yeast SREBP cleavage.

(A) Alignment of glycine-leucine sequence from Sre1 and Sre2 of *Schizosaccharomyces pombe*, and diagrams for Sre1 and Sre2. Numbers indicate respective amino acid positions of Sre1 and Sre2. Arrow indicates predicted cleavage site. Glycine-leucine sequence and predicted transmembrane domains are indicated by red and black boxes, respectively. (B) Western blot probed with anti-FLAG IgG of lysates from indicated *sre2Δ* strains expressing either wild-type Sre2 model substrate (MS) or glycine-leucine sequence mutants. (C) Western blot probed with anti-FLAG IgG of lysates from the indicated strains expressing either wild-type Sre2-MS or cleavage-defective mutants. *P* and *N*, precursor and cleaved nuclear forms, respectively. All strains are *sre2Δ*.

Figure 2.5. Mutants are defective for cleavage of full-length Sre2.

(A) Western blot probed with anti-Sre2 IgG of lysates from the indicated *sre2Δ* strains expressing either full-length wild-type Sre2-GFP or glycine-leucine motif mutants. (B) Western blot probed with anti-Sre2 IgG of lysates from the indicated *sre2Δ* strains expressing either full-length wild-type Sre2-GFP or cleavage-defective mutants. *P* and *N*, precursor and cleaved nuclear forms of Sre2-GFP, respectively. (C) Indicated *sre2Δ* strains expressing either wild-type GFP-Sre2 or GFP-Sre2 mutant were grown in minimal medium lacking leucine and thiamine for 20 hours to induce expression, and imaged by fluorescence microscopy.

Figure 2.6. Glycine-leucine motif is broadly conserved in fungi and functionally conserved in Sre1.

(A) Consensus SREBP glycine-leucine motif in fungi. Consensus sequence logo was generated by aligning 50-amino-acid stretches that were isolated and centered at 55 amino acids before the first predicted TM of fungal SREBPs (see Experimental Procedures for details). Residues required for Sre2 cleavage are boxed. (B) Western blot probed with anti-Sre1 IgG of lysates from the indicated full-length wild-type Sre1 and glycine-leucine motif mutants expressed from the chromosome. Cells were grown in rich medium for the indicated times in the absence of oxygen. Sre1 N408E is used as a control, corresponding to a residue with normal cleavage in Sre2. *P* and *N*, precursor and cleaved nuclear forms of Sre1, respectively. (C) Growth of wild-type, *sre1Δ*, *dsc1Δ*, and indicated *sre1* mutants on YES rich medium containing 1.6 mM cobalt chloride. 5x and 1x indicates 25,000 and 5,000 cells respectively.

Table 2.1

Summary of Sre2 model substrate mutants screened

Cleavage of Sre2 model substrate mutants		
Block (P>N)	Partial (P≈N)	Normal (N>P)
L678T	N679E	V677T
G680E	E685A	V682T
L681T	T686A	T691A/N692A
G683E	V687A/H688A	G697A
L684T	M689A/L690A	S699A
M704A	P693A	R701A
S705A	D694A	F702A/S703A
K743R	D695A	V706A/L707A
E755L	S698A	S710A/P711A
S765L	N700A	S712A/L713A
P767L	P708A	H714L/S715L
D770L	I709A	R718L/F719L
W771L	M731L/H732L	F726L
R778A	S751L	C728L/F729L
E788A	R753L	T736L
	S760L	P737A/E738A
	F761L	T740A/L741A/R742A
	V766L	W744A/S746A
	Y769L	S747A/I748A/Y749A
	N773A	F752L
	Y774A	C756L
	N776A	V757L
	L777A	F758L
	L781A	F764L
	L787A	G772A
		L775A
		G779A
		K780R
		L782A
		L783A
		E784A/L785A/N786A
		S789A
		G790A/V791A
		T793A

Table 2.S1

List of Sre2 model substrate mutants screened	
V677T	T740A/L741A/R742A
L678T	K743R
N679E	K743A
G680E	W744A/S746A
L681T	S747A/I748A/Y749A
V682T	S751L/R753L
G683E	S751L
L684T	F752L
E685A/T686A	R753L
E685A	E755L
T686A	C756L ^a
V687A/H688A	V757L
M689A/L690A	F758L
T691A/N692A	S760L
P693A/D694A	F761L
P693A	F764L
D694A	S765L/P767L
D695A/G697A	S765L
D695A	V766L
G697A	P767L
S698A	Y769L/D770L
S699A	Y769L
N700A/R701A	D770L
N700A	W771L
R701A	G772A/N773A/Y774A
F702A/S703A	G772A
M704A/S705A	N773A
M704A	Y774A
S705A	L775A/N776A
V706A/L707A	L775A
P708A/I709A	N776A
P708A	L777A
I709A	R778A
S710A/P711A	G779A
S712A/L713A	K780R
H714L/S715L	L781A/L782A/L783A
R718L/F719L	L781A
F726L	L782A
C728L/F729L	E784A/L785A/N786A
C728L	L787A/E788A/S789A
F729L	L787A
M731L/H732L	E788A
H732L ^a	S789A
T736L	G790A/V791A
P737A/E738A	T793A

^aCleavage efficiency varied between model substrates (MS): Sre2 MS and GFP Sre2 MS

Table 2.S2

Sequence comparison of fungal SREBP glycine-leucine motifs derived from bioinformatics analysis

Phylum	Organism	Motif
Ascomycota	<i>Schizosaccharomyces pombe</i> Sre1	LNGMVGL
	<i>Schizosaccharomyces pombe</i> Sre2	LNGLVGL
	<i>Schizosaccharomyces japonicus</i> Sre1	LGGMVGL
	<i>Schizosaccharomyces japonicus</i> Sre2	LGGLAGV
	<i>Schizosaccharomyces cryophilus</i> Sre2	LGSLAGL
	<i>Schizosaccharomyces octosporus</i> Sre2	LGGLAGL
	<i>Aspergillus nidulans</i>	LGTLAGL
	<i>Aspergillus fumigatus</i>	LGALAGL
	<i>Aspergillus terreus</i>	LGALAGL
	<i>Verticillium aboatrum</i>	VGSLAGL
	<i>Neosartorya fischeri</i>	LGALAGL
	<i>Glomerella graminicola</i>	VGSLAGL
	<i>Gibberella zeae</i>	VGSLAGL
	<i>Magnaporthe oryzae</i>	VGSLAGL
	<i>Neurospora crassa</i>	VGSLAGL
	<i>Candida albicans</i>	LGGMAAV
	<i>Candida dubliniensis</i>	LGGMAAV
	<i>Pichia angusta</i>	VGDRKGL
	<i>Kluyveromyces lactis</i>	N / A
Basidiomycota	<i>Cryptococcus neoformans</i>	N / A
	<i>Malassezia globosa</i>	N / A
	<i>Ustilago maydis</i>	N / A
	<i>Piriformospora indica</i>	N / A
	<i>Sporisorium reilianum</i>	N / A
	<i>Laccaria bicolor</i>	N / A
	<i>Puccinia graminis f. sp. tritici</i>	N / A
	<i>Moniliophthora perniciosa</i>	N / A

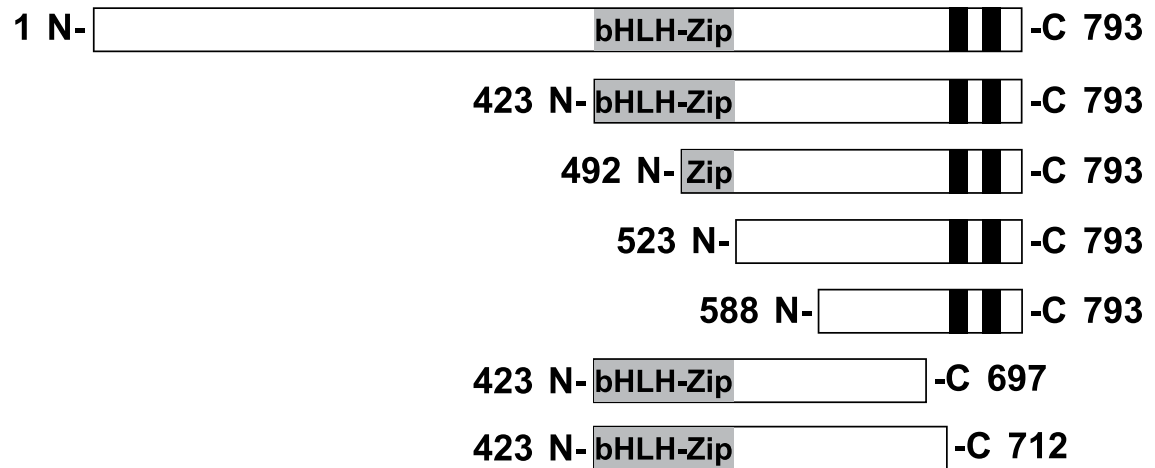
Table 2.S3

Strain table

<i>S. pombe</i> Strains	Genotype	Reference or Source	Figure
PEY553	<i>h- leu1-32 ura4-D18 ade6-M210 his3-D1 Δsre2::kanMX6</i>	Hughes <i>et al.</i> 2005	1
PEY1642	<i>h- leu1-32 ura4-D18 ade6-M210 his3-D1 Δdsc1::kanMX6 Δsre2::kanMX6</i>	This Study	1,2
PEY1643	<i>h- leu1-32 ura4-D18 ade6-M210 his3-D1 Δdsc2::kanMX6 Δsre2::kanMX6</i>	This Study	2
PEY1645	<i>h- leu1-32 ura4-D18 ade6-M210 his3-D1 Δdsc3::kanMX6 Δsre2::kanMX6</i>	This Study	2
PEY1644	<i>h- leu1-32 ura4-D18 ade6-M210 his3-D1 Δdsc4::kanMX6 Δsre2::kanMX6</i>	This Study	2
PEY1646	<i>h- leu1-32 ura4-D18 ade6-M210 his3-D1 Δdsc5::kanMX6 Δsre2::kanMX6</i>	This Study	2
PEY993	<i>h- leu1-32 ura4-D18 ade6-M210;smt0 rpl42::cyhR(sP56Q);mat1m-cyHS/ PEM-2 P392 cdc48-4::natMX6</i>	This Study	2
PEY522	<i>h- leu1-32 ura4-D18 ade6-M210 his3-D1 Δsre1::kanMX6</i>	Hughes <i>et al.</i> 2005	6
PEY1448	<i>h- leu1-32 ura4-D18 ade6-M210 his3-D1 Δdsc1::kanMX6</i>	Stewart <i>et al.</i> 2011	6
PEY1647	<i>h- leu1-32 ura4-D18 ade6-M210 his3-D1::sre1- his3+ Δsre1::kanMX6</i>	This Study	6
PEY1648	<i>h- leu1-32 ura4-D18 ade6-M210 his3-D1::sre1 G409E-his3+ Δsre1::kanMX6</i>	This Study	6
PEY1649	<i>h- leu1-32 ura4-D18 ade6-M210 his3-D1::sre1 M410E-his3+ Δsre1::kanMX6</i>	This Study	6
PEY1650	<i>h- leu1-32 ura4-D18 ade6-M210 his3-D1::sre1 G412E-his3+ Δsre1::kanMX6</i>	This Study	6
PEY1651	<i>h- leu1-32 ura4-D18 ade6-M210 his3-D1::sre1 N408E-his3+ Δsre1::kanMX6</i>	This Study	6
PEY1652	<i>h- leu1-32 ura4-D18 ade6-M210 his3-D1::sre1 L413T-his3+ Δsre1::kanMX6</i>	This Study	6
The <i>ade6-M21?</i> allele is either <i>ade6-M210</i> or <i>ade6-M216</i> .			

Figure 2.1

A Sre2



B Western Blot

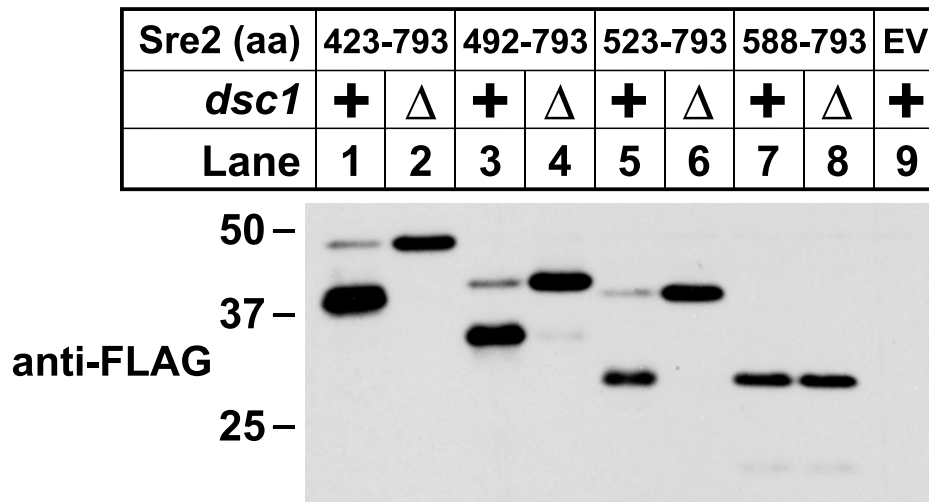
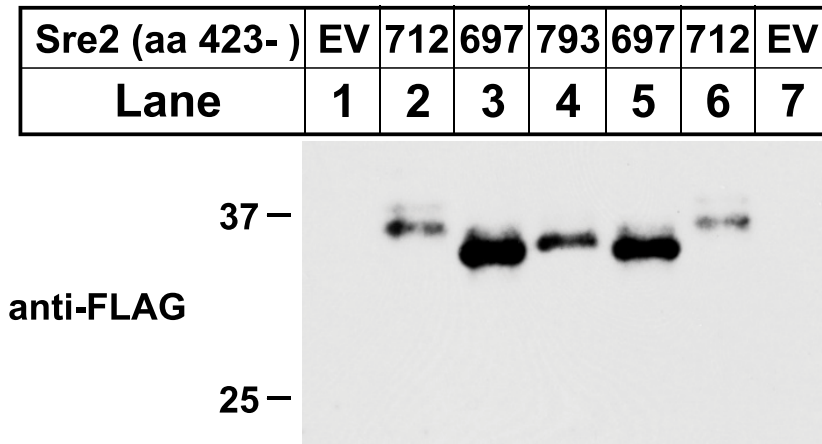
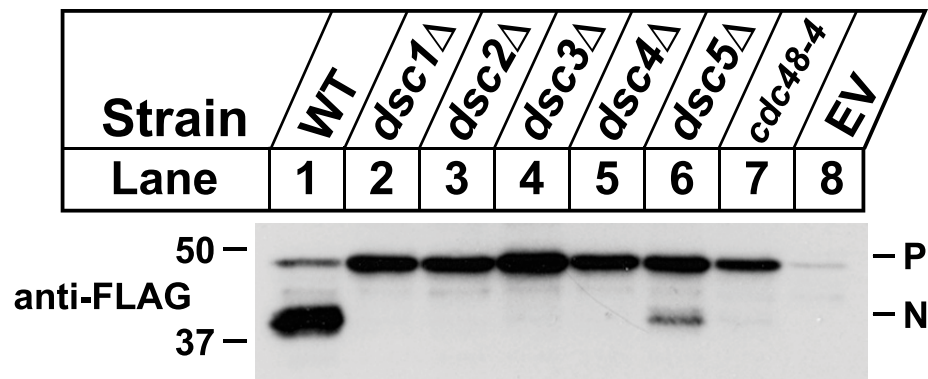


Figure 2.2

A Western Blot



B Western Blot



C Western Blot

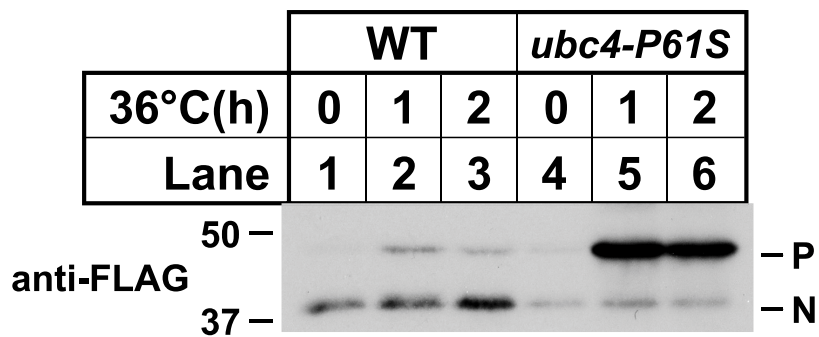
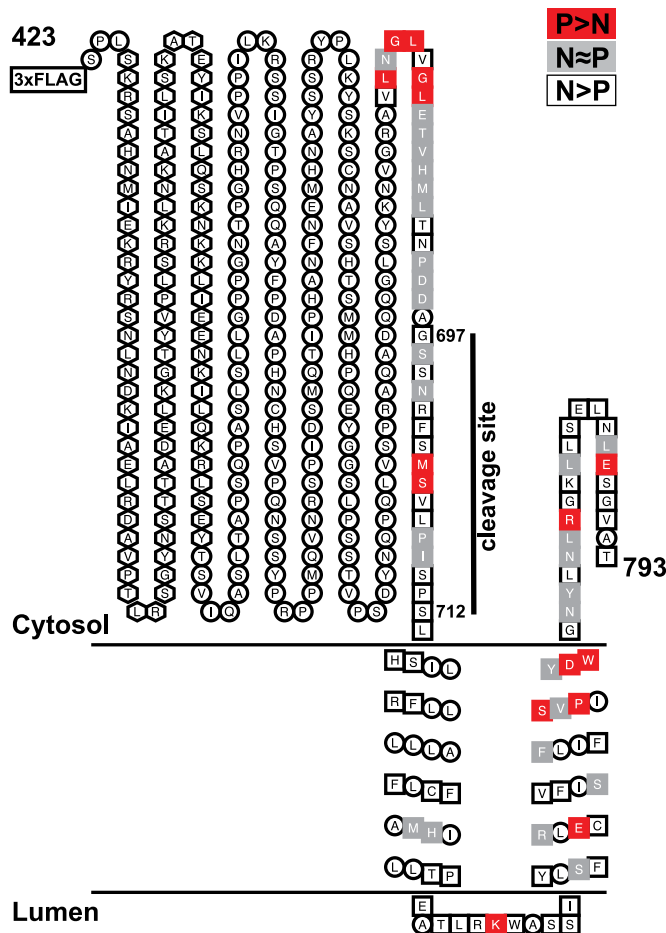


Figure 2.3

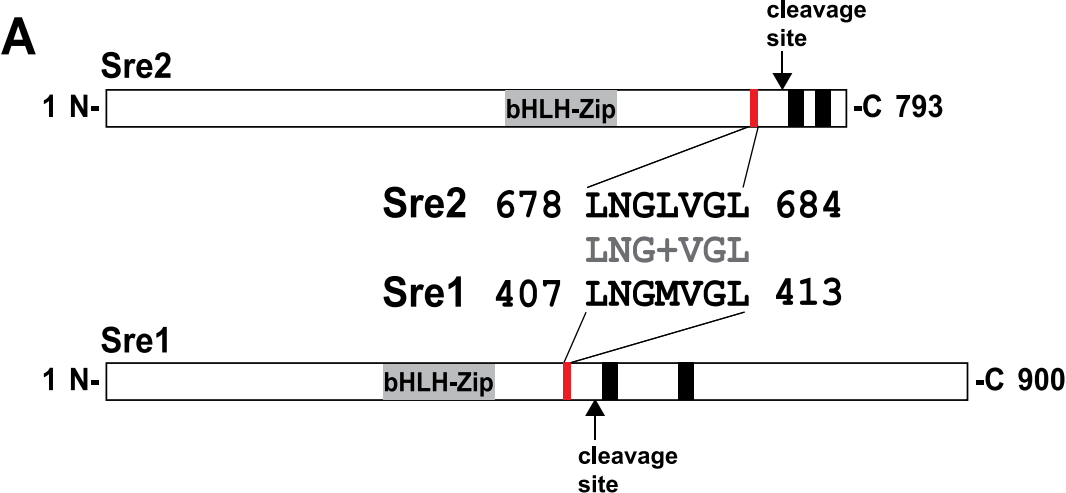
A Sre2 Model Substrate



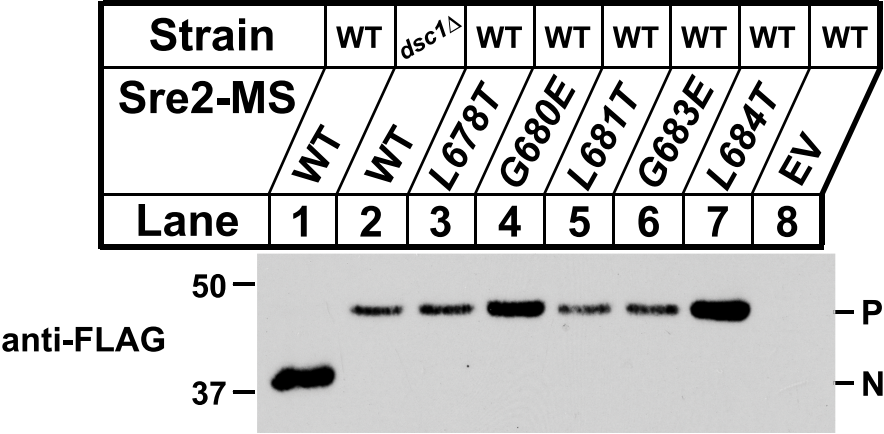
B Western Blot



Figure 2.4



B Western Blot



C Western Blot

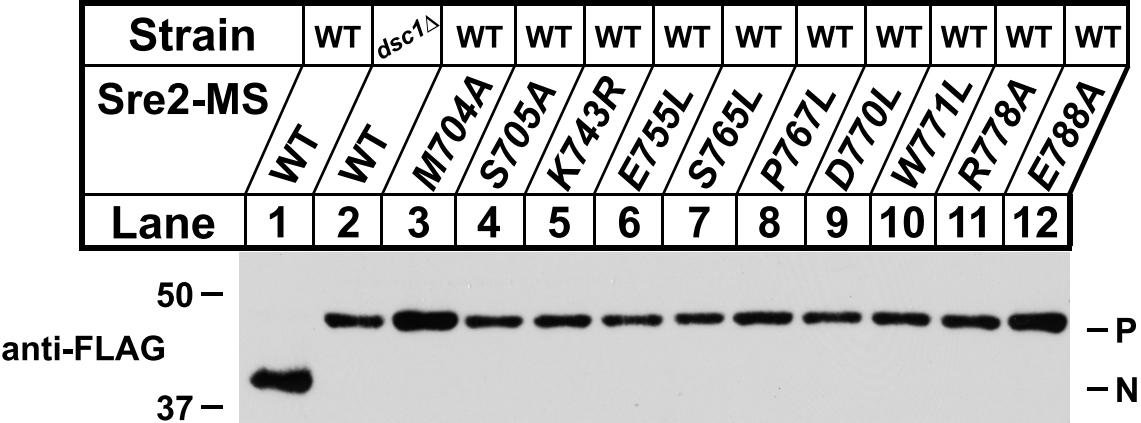
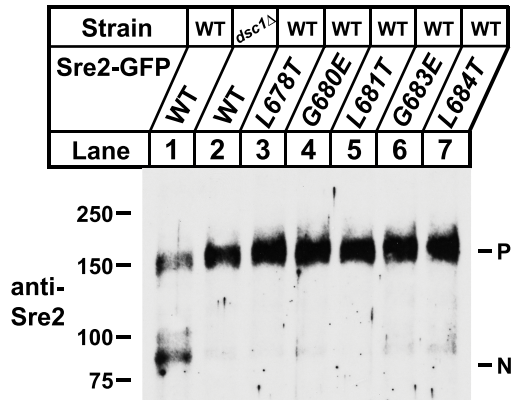
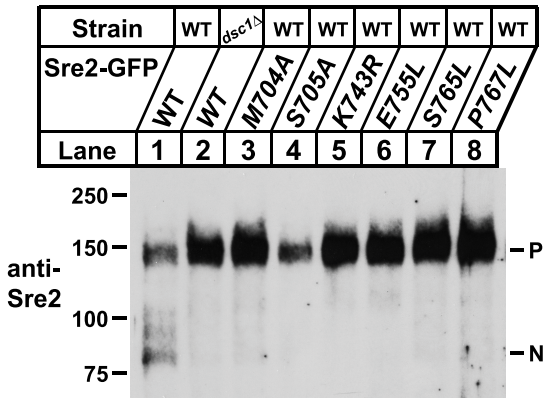


Figure 2.5

A Western Blot



B Western Blot



C

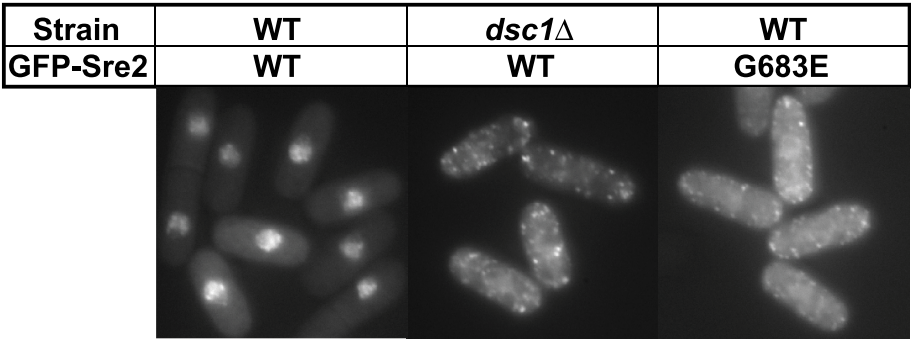
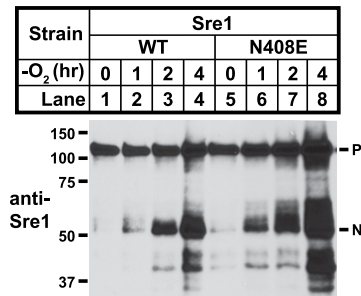


Figure 2.6

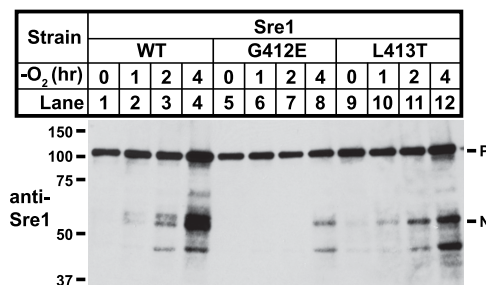
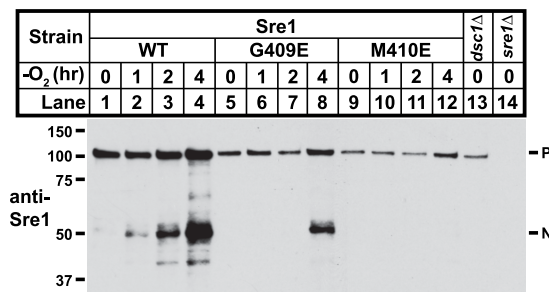
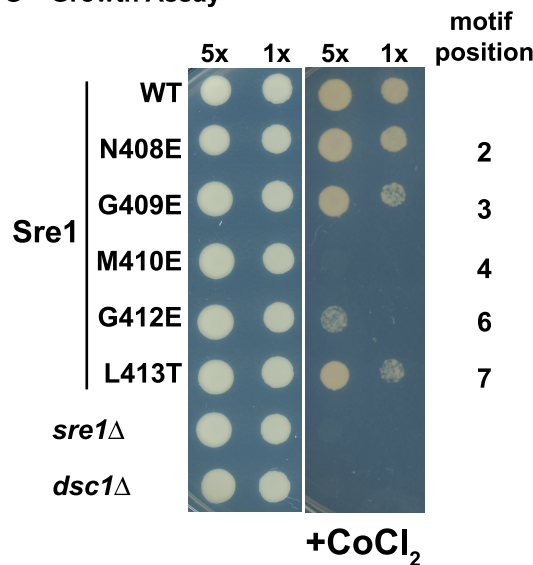
A Fungal SREBP glycine-leucine motif



B Western Blot



C Growth Assay



Chapter 3

Mechanisms of SREBP C-terminal Degradation in Fission Yeast

Summary

Fission yeast SREBPs are proteolytically activated to release the functional N-terminal transcription factor. However, the protease responsible for cleaving SREBP remains elusive. Monitoring the fate of SREBP C-terminus represents a useful vantage point to dissect the elusive yeast SREBP cleavage mechanism. In this chapter, I established Sre2-GFP as a model to study potential C-terminal regulation and degradation of fission yeast SREBPs. I demonstrated that Sre2 C-terminal fragments are degraded in a Dsc E3 ligase dependent manner. This degradation relies on the core subunits Dsc1 through Dsc4, but not the UBX-domain protein Dsc5, the AAA-ATPase Cdc48, or the putative rhomboid protease Rbd2. Moreover, the degradation of these Sre2 C-terminal fragments requires the proteasome in a time-dependent manner. Finally, Dsc1 E3 ubiquitin ligase-dependent degradation of Sre2 C-terminal fragments also does not require Rbd2. While the identities of the SREBP C-terminal fragments await further characterization, these results described mechanisms of C-terminal degradation of SREBPs through the core subunits of Dsc E3 ligase and the proteasome, and represents the first steps towards understanding Golgi membrane protein degradation by means of a physiological protein substrate.

Introduction

Transcription factors can be released from the membrane by two major mechanisms: regulated intramembrane proteolysis (RIP) and regulated ubiquitin/proteasome-dependent processing (RUP) (Hoppe et al., 2001). In the case of RIP, proteases that are embedded in the membrane cleave substrate membrane proteins. For RUP, protein release from the membrane requires the ubiquitin-proteasome system (UPS).

RIP requires one of the three classes of intramembrane proteases: site-2 protease, gamma-secretase, and rhomboid protease (Brown et al., 2000). Site-2 protease is a zinc metalloprotease responsible for cleaving and releasing SREBP from the membrane in mammalian cells (Rawson et al., 1997; Sakai et al., 1996). Gamma-secretase is responsible for the cleavage of amyloid beta-protein precursor APP implicated in Alzheimer's disease (Selkoe, 1994). Rhomboid proteases are a class of universally conserved intramembrane serine proteases that cleave other transmembrane proteins, with *E. coli* Spitz and *Drosophila* epidermal growth factor (EGF) receptor as representative substrates (Lee et al., 2001; Urban and Dickey, 2011).

RUP makes use of the ubiquitin/proteasome system to process cellular protein substrates. Protein substrates are marked by ubiquitin, and can either be mono- or polyubiquitinated (Hershko and Ciechanover, 1998). Polyubiquitinated substrates are destined for degradation by the 26S proteasome (Kim et al., 2011). Alternatively, RUP activates both soluble and membrane transcription factors in mammals and yeast (Rape and Jentsch, 2004). For NFkB, the production of active transcription factor, p50, from the precursor p105 protein requires the 26S proteasome (Palombella et al., 1994). The

budding yeast *S. cerevisiae* possesses two transcription factors/coactivators, Mga2p and Spt23p, requiring the ubiquitin-proteasome system for activation (Hoppe et al., 2000; Piwko and Jentsch, 2006; Rape and Jentsch, 2002). Most recently, Def1, a protein responsible for RNA polymerase II-dependent DNA damage response, is processed in response to transcription stress through ubiquitination and proteasome-dependent processing (Wilson et al., 2013). The processed Def1 fragment accumulates in the nucleus and binds to RNA polymerase II for triggering its degradation (Wilson et al., 2013).

In this chapter, I aimed to use C-terminal degradation of fission yeast SREBP to investigate how SREBPs are processed and to establish fission yeast SREBPs as physiological substrates for Golgi protein processing. Membrane-bound transcription factors have to be released through a regulated manner to control cell homeostasis. Since Site-2 protease is not conserved in fission yeast, what is the mechanism of cleavage in this system? Is fission yeast SREBP released through RIP or RUP? Even though the Dsc E3 ligase is required for yeast SREBP cleavage activation, no active proteases were identified through the initial genetic screens (Stewart et al., 2012; Stewart et al., 2011). Excitingly, recent work from our group identified a fission yeast rhomboid protease Rbd2 as a candidate yeast SREBP protease through a high-throughput genetic experiment (unpublished observation, Diedre Ribbens and Adam Frost). This study aimed to further dissect the potential roles of RIP through this rhomboid protease, and RUP by the proteasome as candidate direct proteolytic mechanisms. This chapter provided important insights into how SREBPs are processed in fungi.

Results

Using Sre2-GFP to monitor Sre2 C-terminal fate

Sre2 is a 793 a.a. protein with two transmembrane segments (a.a. 714-737 and a.a. 749-771) (Figure 3.1A). To track Sre2 cleavage, Sre2 antibody was generated against the N terminus of Sre2, allowing the detection of both the precursor form (P) and the cleaved nuclear form (N). To monitor the fate of Sre2 C-terminus, I inserted GFP at the C-terminus of Sre2, allowing the detection of the C-terminus using GFP antibody. In addition, this also has the benefit of distinguishing Sre2 precursor from Sre2 N-terminal cleaved form (Figure 3.1A), thus allowing me to track both Sre2 N and C termini simultaneously. Importantly, Sre2-GFP allows detailed study of the degradation of Sre2 C-terminus that is dependent upon the Dsc E3 ligase, which is genetically required for cleavage activation of Sre2 (Stewart et al., 2012; Stewart et al., 2011).

Dsc1 E3 ligase-dependent degradation of Sre2 C-terminal fragments

To investigate whether any Sre2 C-terminal products can be detected at steady state, I overexpressed Sre2-GFP in *sre2Δ* cells (otherwise wild-type condition), and probed for any GFP-associated fragments. Sre2 C-terminal fragments were not detectable using GFP antibody, suggesting that it is degraded constantly at steady state (Figure 3.1B, *lanes 1-6*). However, in cells that were also deleted for the Dsc1 E3 ubiquitin ligase, in addition to Sre2 full length precursors, I saw a robust accumulation of two GFP-associated fragments running between 25kDa and 37kDa (Figure 3.1B, *lanes 7-10*), suggesting that degradation of GFP-associated fragments required the Dsc1 E3 ubiquitin

ligase. (The lack of detectable signal in lane 11 of Figure 3.1B might indicate a particular yeast isolate with low protein expression.)

To test whether any of the two bands represent a GFP fragment (~26.9 kDa), or GFP fragment attached to Sre2 C-terminus fragments post-cleavage, I generated protein size standards expressing GFP alone or various C-terminal truncations of GFP with Sre2 C-terminus from a plasmid in *sre2Δ* cells or *dsc1Δ sre2Δ* cells and compared their migration patterns with the two Dsc1 E3 ligase-dependent GFP-associated fragments (Figure 3.1B, lanes 7-10). Results from this line of experiments were difficult to interpret (data not shown), due to seemingly random differential degradation of C-terminal truncation constructs. As a further attempt to resolve these fragments, I also performed membrane fractionation of these C-terminal fragments, and these fragments all segregated in the membrane fraction for the different migrating protein species (data not shown).

However, efforts from such studies suggest that the upper band is likely to represent a Sre2 C-terminal fragment attached to GFP due to its significantly higher molecular weight than GFP alone, while the lower band migrated close in molecular weight to GFP (~26.9 kDa). Taken together, the degradation of these two GFP immunoreactive bands is markedly dependent upon the Dsc1 E3 ligase.

Degradation of Sre2 C-terminal fragments requires Dsc1 through Dsc4, but not Dsc5, Cdc48 or Rbd2

Cleavage of Sre1 and Sre2 requires each of the subunits of the Golgi Dsc E3 ligase complex Dsc1 through Dsc5, AAA-ATPase Cdc48, and the rhomboid protease

Rbd2 (Stewart et al., 2012; Stewart et al., 2011; unpublished observations, Diedre Ribbens). To investigate whether degradation requirements of Sre2 C-terminal fragments parallel those of Sre1 and Sre2 cleavage, I examined the degradation of the C-terminal fragments in cells lacking each of these components. I found that Dsc1 through Dsc4 were strictly required for degradation of Sre2 C-terminal fragments (Figure 3.2, *lanes 2-5*). In *dsc5Δ* cells, Sre2 C-terminal fragments were not detected (Figure 3.2, *lane 6*), consistent with partial requirement of *dsc5* for the cleavage of Sre2 precursor (Stewart et al., 2012). *cdc48-4* contains a mutation (E325K) that lies in the Walker B motif in the AAA-ATPase domain of Cdc48 (Stewart et al., 2012). In these cells, only a small portion of the lower migrating band is detected upon high but not low exposure times of Western blot, reflecting efficient degradation similar to wild-type cells (Figure 3.2, *lane 1*). In cells lacking the rhomboid protease Rbd2, Sre2 C-terminal fragments were also undetected. Further, these results also hold true using an Sre2-GFP plasmid based assay in the indicated deletion or mutant strains (data not shown). Collectively, these results indicate that the requirement for degradation of Sre2 C-terminal fragments require the core Dsc E3 ligase subunits Dsc1 through Dsc4, but not Dsc5, Cdc48 or Rbd2.

Degradation of Sre2 C-terminal fragments requires the proteasome

Because Sre2 C-terminal fragments are unstable in wild-type cells, I next sought to determine the mechanism by which the fragments are degraded. To test whether degradation of the C-terminal fragments in wild-type cells requires the proteasome, wild-type cells were treated with the proteasome inhibitor for 1, 2, 3 or 4 hours and C-terminal fragments were probed using GFP antibody (Figure 3.3, *lanes 1-5*). Treatment with the

proteasome inhibitor bortezomib increased the protein levels of C-terminal fragments only after 1 hour treatment. (The increased protein level in Figure 3.1 *lane 2* compared to *lane 1* was consistent in a separate experiment analogous to Figure 3.1, *lanes 1-5*, for which proteins were assessed as equally loaded by beta-actin control). Interestingly, the dynamics of proteasome stabilization and the protein expression level were distinct for the “upper band” (closest to 37kDa), the “lower band” (closest to 25kDa), and an additional proteasome-dependent band closest to 20kDa. The “upper band” was stabilized maximally at steady state after 1 hour treatment with bortezomib and did not increase over additional time points (Figure 3.3, *lanes 1-5*). In wild-type cells, the proteasome-dependent band that migrated closest to 20kDa follows similar dynamics to the upper band. This band likely represents a degradation product associated with the “upper band”. Also, the “upper band” was present at a much higher level than the “lower band” in the presence of bortezomib. The “lower band”, more accurately described as a closely migrating set of doublets, however, illustrated a time-dependent stabilization that was maximal at 4 hours. While these fragments had differential proteasome-dependent stabilization dynamics, I conclude that degradation of Sre2 C-terminal fragments is dependent upon the proteasome.

In addition, to further probe whether the proteasome also regulates C-terminal degradation in the absence of Dsc1 E3 ligase, the same time course experiment was performed in *dsc1Δ* cells. Besides the appearance of the additional proteasome-dependent band (closest to 20kDa), there was no proteasome-dependent stabilization of any additional C-terminal fragments (Figure 3.3, *lanes 6-10*). These results indicate that Dsc1 E3 ubiquitin ligase-dependent C-terminal fragments are generated through

mechanisms of cleavage independent of proteasome activity, and raises the possibility that these fragments might represent protease cleavage products that are degraded in a Dsc E3 ligase complex-dependent manner.

Dsc1 E3 ubiquitin ligase-dependent degradation of Sre2 C-terminal fragments does not require the rhomboid protease Rbd2

To directly test whether the Dsc1 E3 ubiquitin ligase-dependent fragments are cleavage products of the rhomboid protease Rbd2, I performed epistasis testing to track Sre2 N and C termini in either single mutants *dsc1Δ* and *rbd2Δ* cells, or *dsc1Δ rbd2Δ* double mutant cells. The results from tracking the Sre2 N-terminus suggest that *dsc1Δ* is epistatic to *rbd2Δ* for Sre2 cleavage, assuming a biochemical pathway (Figure 3.4, lanes 1-5, lower panel). C-terminal fragments were only present in *dsc1Δ* cells (Figure 3.4, lane 2, upper panel) but not *rbd2Δ* cells (Figure 3.4, lane 3, upper panel). If either one of the two the C-terminal fragments is/are indeed cleavage product(s) of Rbd2, I would expect the disappearance of one or both of the C-terminal fragments only in *dsc1Δ rbd2Δ* double mutant cells but not in *dsc1Δ* cells. However, both C-terminal fragments were still present in *dsc1Δ rbd2Δ* cells (Figure 3.4, lane 4, upper panel). These results also hold true in an Sre2-GFP plasmid based assay in the respective relevant genetic backgrounds (data not shown). Collectively, these results suggest that Dsc1 E3 ligase-dependent Sre2 C-terminal fragments are not cleavage products of Rbd2.

Discussion

For SREBP cleavage in either fungal or mammalian cells, the fate of the membrane-associated C-terminal fragment post-SREBP cleavage is largely unexplored. In this chapter, I monitored the C-terminal associated fragment(s) of the fission yeast SREBP Sre2 and identified potential mechanisms of Sre2 C-terminal degradation through the Dsc E3 ligase and the proteasome.

Importantly, the identities of the Dsc E3 ligase complex-dependent Sre2 C-terminal fragments await further characterization (Figures 3.1 and 3.2). It is worth noting that the molecular weight of GFP is estimated to be 26.9kDa, while the upper band migrates at a molecular weight significantly larger than 26.9kDa (closer to 37kDa, which represents potentially ~60-70 amino acids of Sre2 C-terminus post-cleavage event). Therefore, it is likely that the upper band represents a product that contains part of the Sre2 C-terminus after Sre2 cleavage. The lower band migrates closer in molecular weight to GFP alone. It is tempting to speculate that the prominence of the lower band compared to the upper band reflects that the lower band is more stable and that the C-terminus of Sre2 is trimmed off to a more stable product that is either GFP alone or a smaller C-terminal Sre2 fragment. Since results from protein size standards or membrane fractionation experiments remain inconclusive, an immediate future direction will be to map the Sre2 cleavage site through protein sequencing or mass spectrometry analysis of both the N-terminal and C-terminal fragments in order to further resolve the identities of these degradation/cleavage fragments.

I tested the genetic requirements for degradation of Sre2 C-terminal fragments. Such degradation requires Dsc1 through Dsc4, but not Dsc5, Cdc48 or the putative

rhomboid protease Rbd2 (Figure 3.2). This result is consistent with Sre2 cleavage requirements for the release of the soluble N-terminus from the membrane, requiring Dsc1 through Dsc4, while only partially requiring Dsc5 and other mutant alleles of Cdc48 (Stewart et al., 2012; Stewart et al., 2011). Consistent with biochemical data of the subunit architecture of the Dsc E3 ligase, Dsc1 through Dsc4 constitutes the core subunits of the multi-subunit Dsc E3 ligase, while Dsc5 and Cdc48 might play a more indirect biochemical or mechanistic role in Sre1 and Sre2 cleavage (Lloyd et al., 2013; Stewart et al., 2012).

I also investigated mechanisms underlying the degradation of Sre2 C-terminal fragments. I found that in wild-type cells, the fragments are rapidly degraded by the proteasome in a time-dependent manner (Figure 3.3). However, in cells that lack Dsc1 E3 ubiquitin ligase, the migrating patterns of the C-terminal fragments remained similar. These might suggest that the Dsc1 E3 ligase-dependent degradation products are terminal, i.e. the proteasome does not cleave SREBP to release a higher molecular weight or stabilized C-terminal product(s) upon inhibition of proteasome function. If the proteasome is the protease responsible for yeast SREBP cleavage, we might expect a larger C-terminal associated product at steady state. The lack of proteasome-dependent stabilization might lead us to speculate that the proteasome is not likely to be the sole proteolytic machinery responsible for cleaving SREBP precursors to release functional SREBPs in fission yeast, similar to the cleavage activation of Mga2 or Spt23 (Hoppe et al., 2000; Rape et al., 2001).

Finally, Dsc1 E3 ubiquitin ligase-dependent Sre2 C-terminal fragment(s) degradation also does not rely on the putative fission yeast rhomboid protease Rbd2

(Figure 3.4). Currently, Rbd2 is shown to be genetically required for Sre1 and Sre2 cleavage (unpublished observations), while whether Rbd2 is directly cleaving Sre2, and if so, the site and mechanism of cleavage remain elusive. Nonetheless, these experiments uncovered and described the potential C-terminal degradation of SREBPs through the core subunits of Dsc E3 ligase and the proteasome, and provide important insights for SREBP and Golgi protein processing.

Experimental Procedures

Materials

We obtained yeast extract, peptone and agar from BD Biosciences; Edinburgh minimal medium (EMM) from MP Biomedical; oligonucleotides from Integrated DNA Technologies; alkaline phosphatase from Roche; bortezomib from LC Laboratories; HRP-conjugated, affinity purified donkey anti-rabbit and anti-mouse IgG from Jackson ImmunoResearch; prestained protein standards from Biorad.

Strains and media

Wild-type haploid *S. pombe* KGY425 and derived strains were grown to log phase at 30°C in YES medium (5 g/liter yeast extract plus 30 g/liter glucose and supplements, 225 mg/liter each of adenine, uracil, leucine, histidine, and lysine) or EMM plus supplements unless otherwise indicated. Yeast transformations were performed as previously described (Hughes et al., 2005). Strains used in this study were derived using standard genetic techniques (Bahler et al., 1998).

Antibodies

We obtained anti-GFP (a mixture of clones 7.1 and 13.1) from Roche. Antisera to Sre2 (a.a. 1-426) polyclonal IgG generated against the cytosolic N-terminus of fission yeast Sre2 has been described previously (Hughes et al., 2005).

Plasmids

RCP23 encodes Sre2-GFP (a.a. 1-1026) under control of the constitutive cauliflower mosaic virus (CaMV) promoter derived from pSLF101 (Forsburg, 1993).

Cleavage assay

For Sre2 cleavage assay, whole cell lysates were prepared for immunoblotting analysis. Protein preparation and immunoblotting for *S. pombe* experiments were described previously (Hughes et al., 2005). For Sre2, whole cell lysates were extracted and treated with alkaline phosphatase as described previously (Hughes et al., 2005). For Sre2-GFP, anti-Sre2 polyclonal antibody was used to detect the precursor (P) and cleaved N-terminal nuclear form (N).

Acknowledgments

I am grateful to Diedre Ribbens for unpublished observations regarding the rhomboid protease Rbd2 and for Rbd2-related strains. Financial support for this work was provided by National Institutes of Health grant HL077588 (P.J.E.) and an Isaac Morris Hay and Lucille Elizabeth Hay Graduate Fellowship Award (R.C.).

Author Contributions

R.C. performed all experiments. R.C. and P.J.E. designed the study.

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Figure legends

Figure 3.1 Dsc1 E3 ubiquitin ligase dependent degradation of Sre2 C-terminal fragments.

(A) Schematic diagram of Sre2 and Sre2-GFP. Predicted transmembrane domains are indicated by black boxes. GFP is appended at the C-terminus of Sre2 to monitor its fate. Basic helix-loop-helix zipper domain (bHLH-zip) and GFP are indicated by gray boxes.

(B) Western blot probed with anti-GFP IgG of lysates. Sre2-GFP is expressed under the control of CaMV promoter on a plasmid for the indicated strains. Multiple isolated are tested for each genotype as tested. Empty vector (EV) for wild-type is included as control for antibody specificity. All strains are *sre2Δ*.

Figure 3.2 Degradation Of Sre2 C-terminal fragments requires Dsc1 through Dsc4 but not Dsc5, Cdc48 or the rhomboid protease Rbd2.

Western blot probed with anti-GFP and anti-Sre2 IgG of whole cell lysates for the indicated strains. GFP is attached to the Sre2 C-terminus at its endogenous locus for the wild-type and indicated deletion or mutant strains. *P* and *N*, precursor and cleaved nuclear forms, respectively.

Figure 3.3 Time-dependent degradation of Sre2 C-terminal fragments by the proteasome.

Western blot probed with anti-GFP and anti-Sre2 IgG of whole cell lysates. The proteasome inhibitor bortezomib (2 mM) is added to the cells at a time course of 0, 1, 2, 3 or 4 hrs before they are harvested for protein analysis. Sre2-GFP is expressed from a

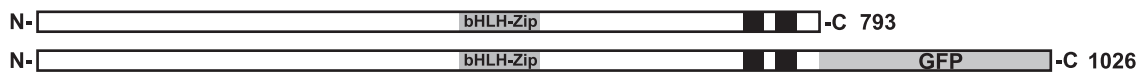
plasmid under the control of CaMV promoter for the indicated strains. Empty vector (EV) for wild-type and *dsc1Δ* is included as control for antibody specificity. All strains are *sre2Δ*. *P* and *N*, precursor and cleaved nuclear forms, respectively.

Figure 3.4 Dsc1 E3 ubiquitin ligase-dependent degradation of Sre2 C-terminal fragments does not require the rhomboid protease Rbd2.

Western blot probed with anti-GFP and anti-Sre2 IgG of whole cell lysates. GFP is attached to the Sre2 C-terminus at its endogenous locus for the wild-type and indicated deletion or mutant strains. *P* and *N*, precursor and cleaved nuclear forms, respectively.

Figure 3.1

A Sre2 and Sre2-GFP



B Western Blot: Sre2-GFP Plasmid

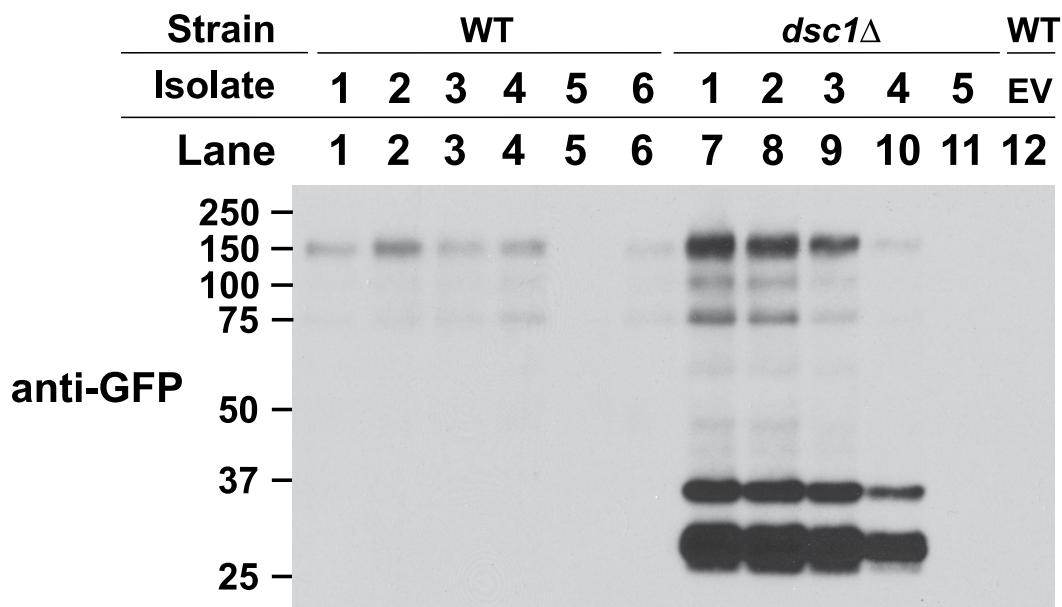


Figure 3.2

Western Blot: Sre2-GFP strains

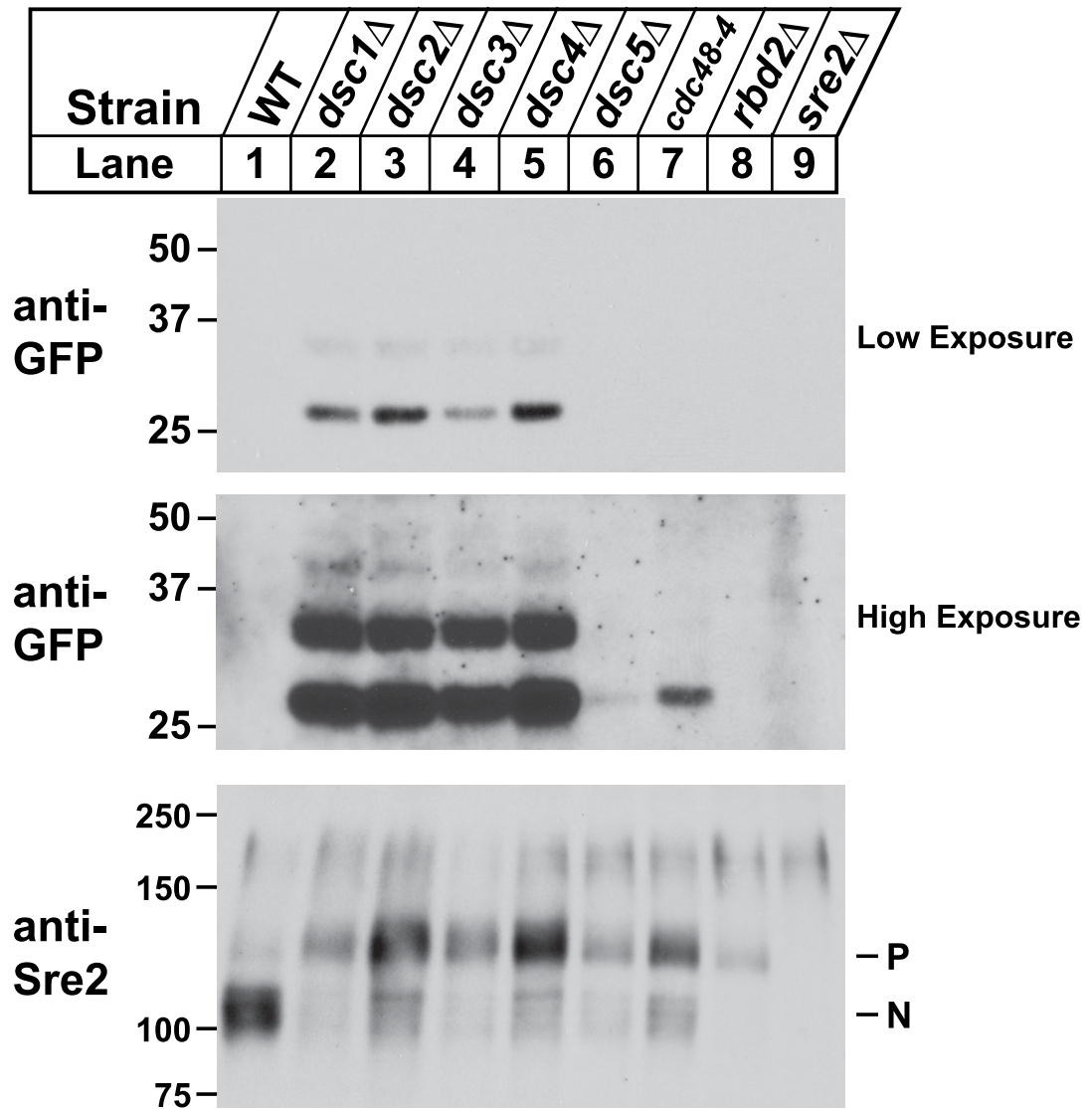


Figure 3.3

Western Blot: Sre2-GFP Plasmid

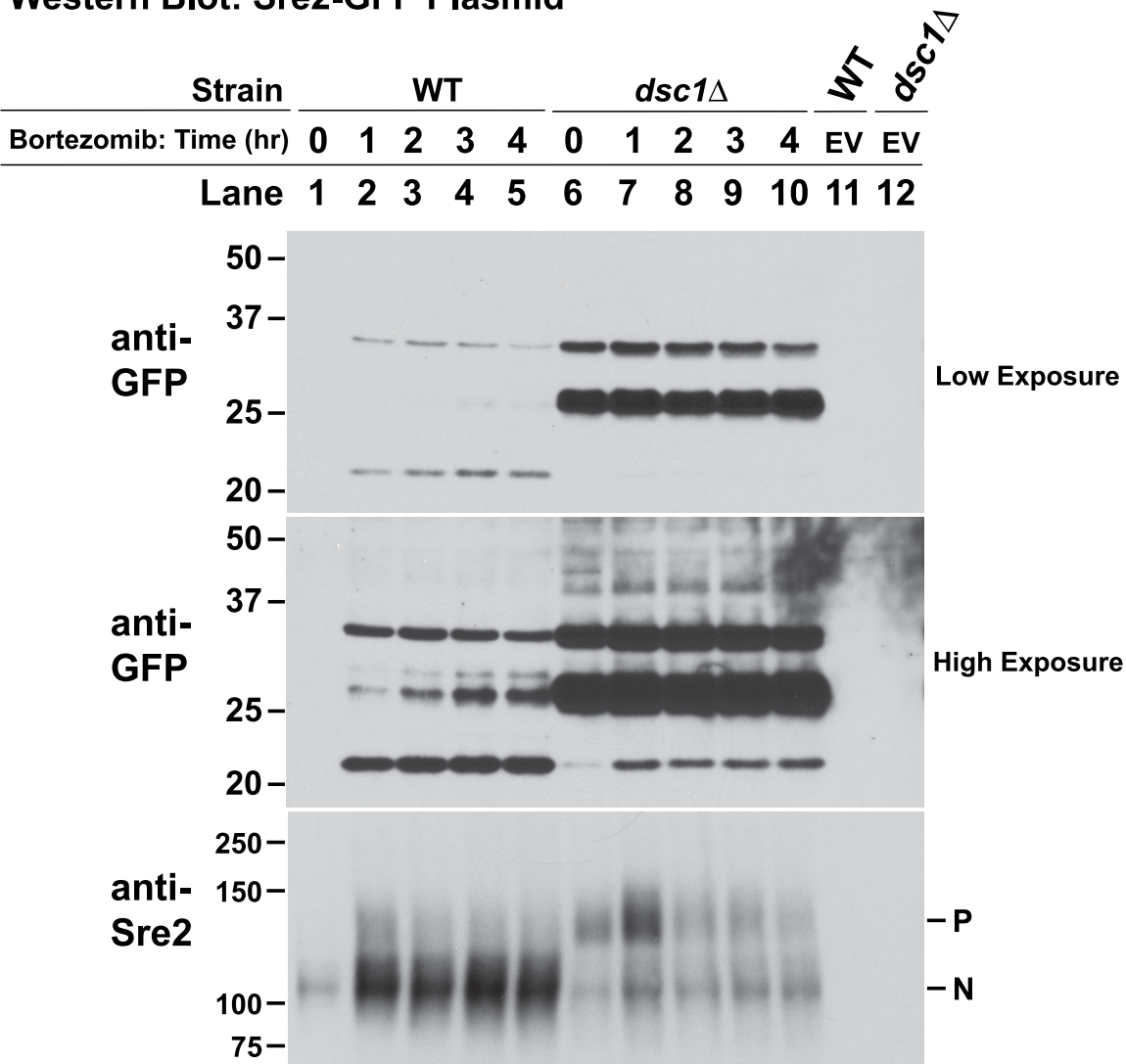
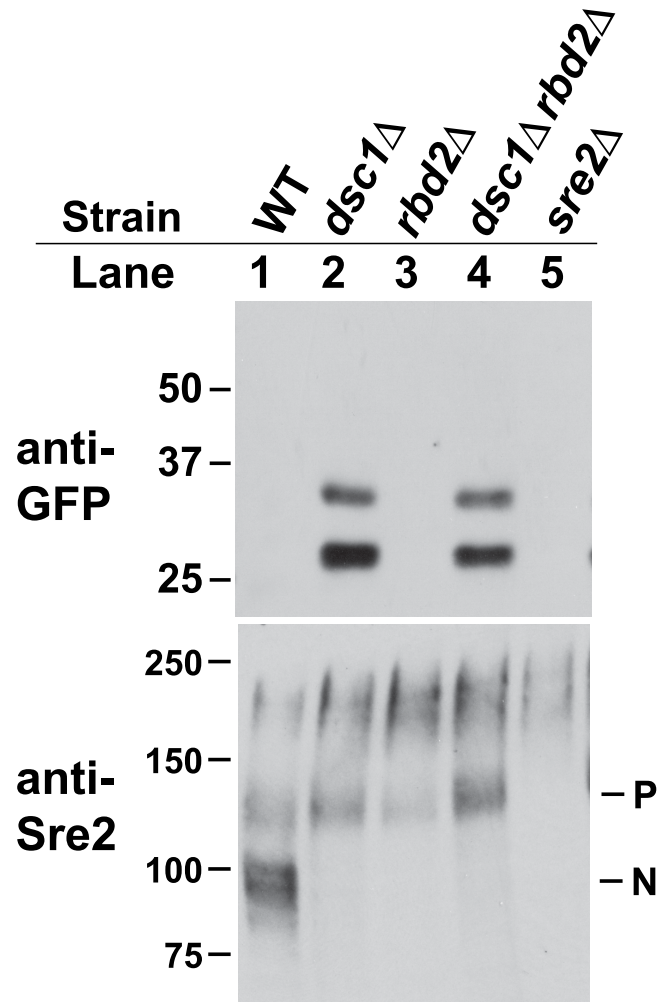


Figure 3.4

Western Blot: Sre2-GFP Strains



Chapter 4

Conclusions

Thesis Summary

In my graduate work, I defined structural requirements for yeast SREBP cleavage (Cheung and Espenshade, 2013), and investigated degradation of Sre2 C-terminus to delineate the mechanism of fungal SREBP cleavage. Sre2 cleavage mutants isolated from my study may be blocked in the following cleavage steps (Figure 4.1): (1) Sre2 transport from the ER to Golgi; (2) Sre2 binding to any subunits of the Dsc E3 ligase; (3) Sre2 ubiquitination by the E2 ubiquitin-conjugating enzyme Ubc4 and E3 ligase Dsc1. Alternatively, these mutants can also be defective in mechanistic steps involving other components of the pathway, such as the candidate protease putative rhomboid protease Rbd2 identified from our recent work, which facilitates release of the N-terminal transcription factor domain from the membrane.

Furthermore, monitoring the Sre2 C-terminus and deciphering its degradation mechanisms provide further insight into modes of transcription factor cleavage and activation by regulated intramembrane proteolysis (RIP) through the rhomboid protease Rbd2 or regulated ubiquitin-proteasome proteolysis (RUP) by the proteasome. In sum, I have established the fission yeast SREBP Sre2 as an important physiological substrate in the post-ER compartments/Golgi apparatus for degradation and/or processing. These studies will further our understanding of the role of SREBPs in fungal virulence, and cellular oxygen and sterol homeostasis. Since SREBPs are required for fungal pathogenesis, chemical inhibitors can be developed to block this pathway. Therefore, mechanistic insights into how SREBPs are activated could result in novel antifungal therapies.

Final Thoughts

Given genetic requirements of a multi-subunit Golgi Dsc E3 ligase for yeast SREBP activation, current efforts are directed towards understanding the mechanism of SREBP cleavage in this system. Protease components are lacking in the system thus far, besides a genetic requirement for the 26S proteasome for Sre1 cleavage (Stewart et al., 2011), until the recent discovery of the rhomboid protease Rbd2 through high-throughput genetic studies from our laboratory.

Mammalian and basidiomycete fungal mammalian SREBP cleavage require Site-2 protease, while ascomycete fungal SREBP cleavage genetically requires the rhomboid protease and the proteasome. Site-2 protease is not present in all the ascomycete fungi examined, suggesting that different proteases might have co-evolved to release the functional SREBP transcription factor from the membrane in diverse evolutionary lineages. It will be interesting to see whether this theme holds true for other evolutionarily conserved membrane-bound transcription factors and proteases.

Sre2 cleavage does not require its basic helix-loop-helix zipper transcription factor domain, and a minimal substrate of 271 amino acids is still cleaved. This result indicates that the functions of Sre2 as a transcription factor and a substrate for Dsc-dependent cleavage are separable. Mutants blocked for Sre2 cleavage uncovered in this thesis were focused on Sre2 a.a. 673 – 793, from which I identified 15 residues required for cleavage of full-length Sre2 and uncovered a novel SREBP cleavage motif. Additional cleavage mutants and motifs may be located between a.a. 523 – 676, a region required for cleavage but not subjected to site-directed mutagenesis.

Future studies should focus on the following questions: (1) *What is the SREBP protease?* (i.e. Is yeast SREBP cleaved directly by the candidate rhomboid protease Rbd2 by RIP and/or the proteasome by RUP?) Both careful *in vitro* and *in vivo* studies are needed to prove functional relevance and direct protease activity on the substrate. Further characterization of the *in vivo* cleavage sites of Sre2 and fate mapping studies of Sre2 C-terminus in this thesis will also facilitate such dissection.

(2) *Does Dsc E3 ligase ubiquitinate yeast SREBPs?* Thus far, we have been unable to detect stable mono- or polyubiquitinated SREBP species. Previous studies have captured a specific yet transient interaction between the Dsc2 subunit of the Dsc E3 ligase and Sre2 (Stewart et al., 2011), suggesting that the E3 ligase-substrate interaction is not stable. Using an improved protein interaction assay to trap substrate interaction by blocking E2 ubiquitin-conjugating enzyme Ubc4 (unpublished result, Sumana Raychaudhuri), all the cleavage mutants uncovered in my screen are still able to bind to Dsc2 (unpublished results, data not shown). Indeed, more sensitive protein interaction assays needs to be developed under appropriate genetic backgrounds, or additional escorting factors or mechanistic steps await discovery. Alternatively, the Dsc E3 ligase does not act directly on SREBP. Intriguingly, my results on the degradation of Sre2 C-terminal fragments suggest that the Sre2 C-terminus may be ubiquitinated and degraded in a proteasome and Dsc E3 ligase-dependent manner. Studies of Sre2 C-terminus represent fruitful avenues for future investigation.

This leads to the third major question: (3) *What are other Dsc substrates?* Given that budding yeast contains functional Dsc component(s), but does not contain SREBPs (Bien and Espenshade, 2010), Dsc must have a role beyond SREBP cleavage. As a first

pass, other protein substrates in the fungal genomes that contain the fungal glycine-leucine motif can be isolated using bioinformatic approaches and may warrant further investigation as candidate Dsc substrates (Cheung and Espenshade, 2013). Importantly, substrates that are differentially ubiquitinated by the Dsc E3 ligase may be identified by proteomic approaches (Lee et al., 2011).

Precedence exists for post-ER protein degradation, but the molecular machinery remains largely unknown (Arvan et al., 2002). Given the functional parallels between ERAD protein quality control components and the Dsc E3 ligase components, Dsc E3 ligase is a candidate machinery for Golgi protein quality control. Future studies of Dsc E3 ligase-dependent SREBP processing will provide important insights into Golgi protein processing in a relevant physiological context.

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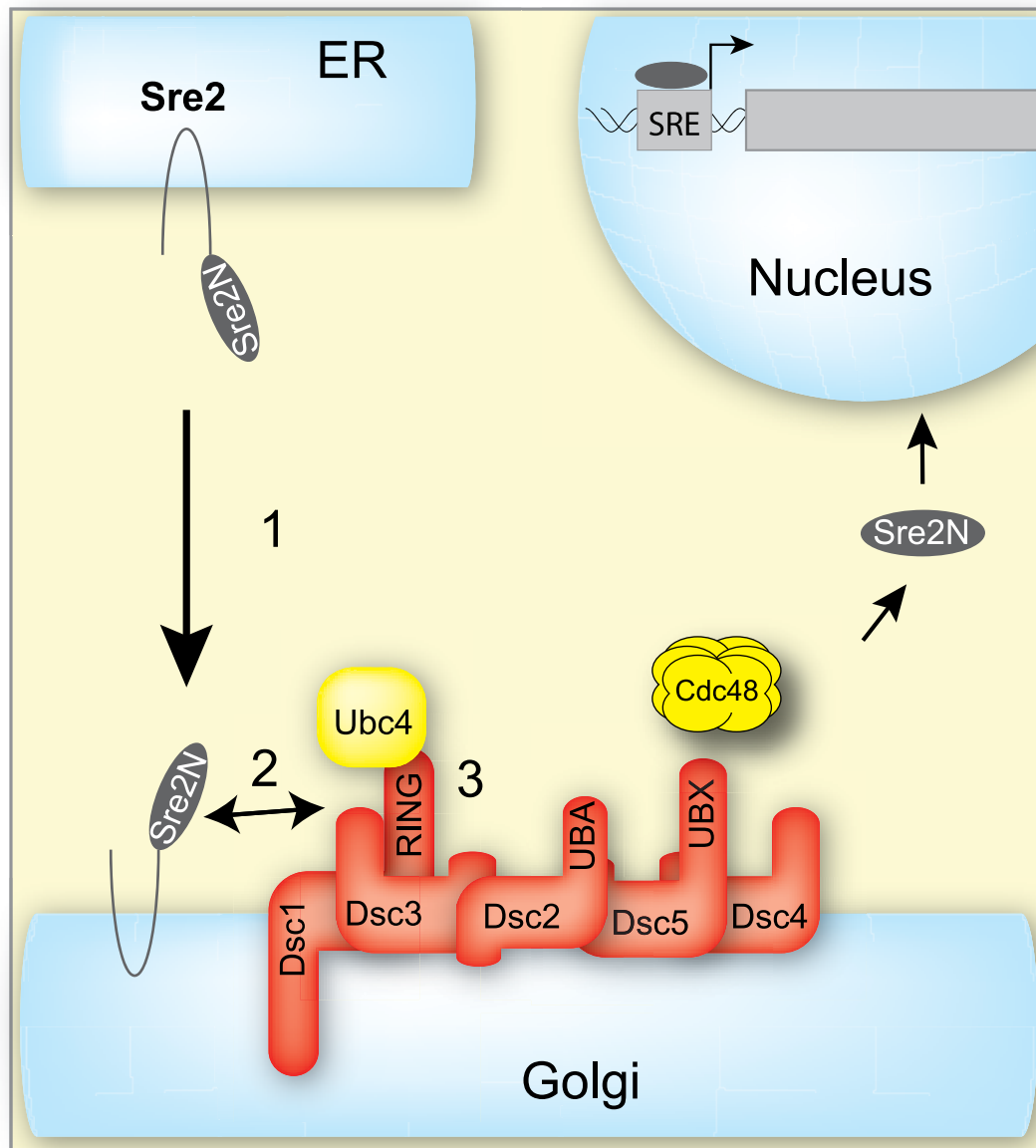
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Figure Legends

Figure 4.1 Mechanistic steps at which cleavage mutants may be defective in fission yeast SREBP cleavage.

Major mechanistic steps at which cleavage mutants can be blocked are denoted numerically in the schematic: (1) transport from endoplasmic reticulum to Golgi; (2) interaction with each of the subunits of the Golgi Dsc E3 ligase; and (3) subsequent ubiquitination of SREBPs by the Dsc E3 ligase. Alternatively, the mutants can block downstream mechanistic steps such as interaction with the putative protease or Cdc48.

Figure 4.1



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Yokota, Y., **Cheung, R.***, Ring, C.*, Pevny, L., and Anton, E.S. Nap1-regulated neuronal cytoskeletal dynamics is essential for the final differentiation of neurons in cerebral cortex. *Neuron*. 2007 May 3;54(3):429-45.

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Sea Urchin Genome Sequencing Consortium [**Cheung, R.** as one of the co-authors]. The genome of the sea urchin *Strongylocentrotus purpuratus*. *Science*. 2006 Nov 10;314(5801):941-52.

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